ONTOGENY OF THERMOTOLERANCE IN BOVINE EMBRYOS: HSP70 SYNTHESIS AND RESISTANCE TO HEAT SHOCK DURING OOCYTE MATURATION AND PREIMPLANTATION DEVELOPMENT

BY

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DEDICATION

This dissertation is dedicated to my family:

* my mother, Janice H. Alberd
* my father, Billy Edwards
* my sisters, Kimberley M. Edwards and Stephanie L. Jordon
* my grandmother, Ozella Edwards
* my great aunt, Ora Lee Coleman
* William B. Howell

Their endless support, encouragement and love sustained me throughout my academic career. A special dedication is made to my mother, Janice H. Alberd. In face of adversity, she always prevails. Without her continued faith, encouragement and optimism the obtainment of one of my many goals, my doctorate, would not have been possible.

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ABBREVIATIONS

1-D SDS-PAGE	1-Dimensional sodium doedecyl sulfate polyacryalmide gel electrophoresis
1-S SDS-PAGE	2-Dimensional sodium doedecyl sulfate polyacryalmide gel electrophoresis
μg	Microgram
ABAM	Antibiotic-antimycotic solution
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphate hydroxylases
BRLC	Buffalo rat liver cells
BSA	Bovine serum albumin
BSO	D,L-buthionine-[S,R]-sulfoximine
BSS	Bovine steer serum
°C	Degrees Centigrade
d	Day
DAPI	4', 6'-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
EGA	Embryonic genome activation
EtBr	Ethidium bromide
ETOH	Ethanol
FDA	Fluoroscein diacetate
FSH-P	Follicle-stimulating hormone (pituitary-derived)
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
h	Hour
hCG	Human chorionic gonadotropin
H_2O	Water
H_2O_2	Hydrogen peroxide
hpi	Hours post insemination
HSE	Heat shock element
HSC	Heat shock cognate protein
HSC70	Heat shock cognate 70
HSC71	Heat shock cognate 71
HSF	Heat shock transcription factor
HSP	Heat shock protein
HSP68	Heat shock protein 68
HSP70	Heat shock protein 70
IFN-τ	Interferon-τ

IU	International unit
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
kDa	Kilodaltons
L	Liter
LH	Luteinizing hormone
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
mRNA	Messenger ribonucleic acid
$\bullet O_2^-$	Superoxide radical
•OH	Hydroxyl radical
OMM	Oocyte maturation medium
P70	Putative heat shock protein 70
P71	Putative heat shock protein 71
PGFM	13,14-dihydro-15-Keto PGF2α
Pen/Strep	Penicillin/streptomycin
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
pI	Isoelectric point
PMSG	Pregnant mare serum gonadotropin
SEM	Standard error of the mean
TALP	Modified Tyrode's solutions
TCA	Trichloroacetic acid
v/v	Volume/volume
wt	Weight
vol	Volume
w/v	Weight/volume

Abstract of Dissertation Presented to the Graduate School
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Requirements for the Degree of Doctor of Philosophy

ONTOGENY OF THERMOTOLERANCE IN BOVINE EMBRYOS: HSP70 SYNTHESIS AND RESISTANCE TO HEAT SHOCK OF OOCYTES AND PREIMPLANTATION EMBRYOS

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Mammalian embryos acquire the ability to withstand elevations in temperature as they proceed in development. The focus of this dissertation was to further characterize the effects of heat shock on the oocyte and early embryo, with the specific intention of identifying potential thermoprotective mechanisms that are involved in protection of preimplantation embryos from deleterious effects of heat shock. Data described in this dissertation demonstrate that environmental signals can activate certain genes before embryonic genome activation usually occurs. Bovine embryos as early as the 2-cell stage can respond to heat shock by modifying the synthesis of a specific protein, HSP68, suggesting that heat shock induces alterations in translation, mRNA stability or transcription of the early embryo. By the 4-cell stage, heat-induced synthesis of HSP68 is the result of alterations in transcriptional activity.

The oocyte and early embryo undergo a biphasic developmental pattern of resistance to heat shock. Two-cell embryos were more sensitive to elevated temperatures than oocytes, suggesting a loss of thermoprotective mechanisms following fertilization and cleavage. Differences in thermal sensitivity of embryos were noted as early as the second cleavage division. This is followed by restoration of resistance to elevated temperature by the morula stage of development. Increased sensitivity of cleavage stage embryos to elevated temperature was not related to an inability to synthesize increased amounts of the thermoprotective molecule, HSP68, following heat shock. Data also suggested a thermoprotective role of cumulus cells and a requirement for GSH during oocyte maturation. Exposure of oocytes to heat shock reduced synthesis of intracellular proteins; percent reduction was greater if cumulus were removed prior to treatment. Moreover, cumulus removal decreased synthesis of HSP70. Inhibition of GSH synthesis during maturation reduced subsequent development of oocytes following fertilization. In conclusion, early embryos can respond to changes in their environment as early as the 2-cell stage by modifying the synthesis of specific proteins. Infertility caused by heat stress may in part be due to effects of elevated temperatures on the early embryo; the decline in deleterious effects of maternal heat stress as pregnancy proceeds is related to differences in the thermal sensitivity of the embryo. Intracellular thermoprotective mechanisms responsible for developmental acquisition of thermotolerance remain undefined but probably include HSP70 and other thermoprotective molecules.

CHAPTER 1
INTRODUCTION

Exposure of females to elevated environmental temperatures results in periods of transient infertility (Poston et al., 1962; Gwazdauskas et al., 1973; Rosenburg et al., 1977; Cavestany et al., 1985). Of all the domesticated animals, deleterious effects of heat stress are most pronounced in lactating dairy cattle because their high metabolic rate causes upper critical temperature (i.e., ambient temperature at which rectal temperature increases) to be low (Fuquay, 1986). As a result, there are very pronounced periods of high embryonic mortality in dairy cattle associated with heat stress. This results in direct economic losses to the producer. Effects of heat stress appear to be more prominent when occurring at or near the time of estrus (Stott & Williams, 1962; Gwazdauskas et al., 1973; Putney et al., 1989a) and may be due to indirect effects on the maternal environment (Howell et al., 1994; Geisert et al., 1988) or direct effects (Ealy et al., 1995) on the oocyte or the early embryo. Interestingly, as embryos proceed in development their ability to withstand elevations in temperature increases (Ealy et al., 1993). Potential thermoprotective mechanisms involved in developmental acquisition of thermotolerance remain unknown; however, correlative evidence in the mouse embryo suggests a role for heat shock protein 70 (HSP70) and glutathione (GSH) (Muller et al., 1985; Hahnel et al., 1986; Gardiner & Reed, 1994; 1995).

A major focus of this dissertation has been to further characterize the effects of heat shock on the oocyte and early embryo, with the specific intention of identifying

potential thermoprotective mechanisms that are involved in protection of early stage embryos from deleterious effects of heat shock. Identification of such mechanisms could result in novel methods for protecting embryos from elevated temperatures. Experiments were designed to 1) determine effects of heat shock on bovine oocytes by evaluating membrane integrity, intracellular synthesis of proteins and developmental potential, 2) characterize the effects of heat shock on early embryos and further define the period during preimplantation development when bovine embryos first gain the ability to withstand elevations in temperature, 3) determine if production of heat-inducible HSP70 molecules is coincident with when induced thermotolerance is first observed in murine embryos (Ealy & Hansen, 1994), and 4) determine the ontogeny of HSP70 synthesis in bovine embryos.

CHAPTER 2
REVIEW OF LITERATURE

In face of a hostile environment, cellular function can be disrupted. For continued survival, cells must rely on adaptive mechanisms that allow them to respond at both the cellular and molecular level. The oocyte and early cleavage stage embryo may be particularly susceptible to disruption by environmental perturbations, because just like a few other cell types (red blood cell), they are unable to transcribe new mRNA. Only following activation of the embryonic genome do embryos acquire the full range of cellular adaptive mechanisms. The following sections are presented to review information pertaining to effects of heat stress on embryonic mortality. Particular emphasis will be placed on the role that one cellular adaptive response, HSP70, plays in cellular thermotolerance. Factors involved in timing of the regulation of embryonic genome activation will also be discussed because of the potential importance for the timing of embryonic genome activation (EGA) in establishment of embryonic thermotolerance.

Effects of Elevated Temperature on Embryonic Mortality in Dairy Cattle

Deleterious effects of heat stress on reproductive processes have been well described in domesticated animals. In conjunction with high humidity, elevated ambient temperature impairs the ability of the animal to balance heat loss with heat gained from the environment and metabolic functions associated with lactation, growth and maintenance (Fuquay, 1986). Periods of heat stress are associated with reduced

expression of estrus (Gangwar et al., 1965; Roller & Stombaugh, 1974; Wolff and Monty, 1974; Gwazdauskas et al., 1981; Thatcher & Collier, 1986; Wolfenson et al., 1988; Ealy et al., 1994) and pregnancy rates in dairy cattle (Stott, 1960; Poston et al., 1962; Stott & Williams, 1962; Dunlap & Vincent, 1971; Ingraham et al., 1974; Monty & Wolff, 1974; Badinga et al., 1985; Cavestany et al., 1985), mice (Elliott et al., 1968; Bellve, 1972; 1973), rabbits (Ulberg & Sheean, 1973; Wolfenson & Blum, 1988), pigs (Warnick et al., 1965; Tompkins et al., 1967) and sheep (Dutt et al., 1959; Dutt, 1963; Woody & Ulberg, 1964). Work in sheep demonstrated that decreased fertility during periods of heat stress is due to increased embryonic mortality rather than fertilization failure (Alliston & Ulberg, 1961). Similar findings have been reported for the bovine (Putney et al., 1988a; 1989a). The following is a summary of seasonal associated increases in embryonic mortality in dairy cattle, specifically describing heat-induced alterations in the maternal environment and direct effects of elevated temperature on a variety of cell types including gametes and the early embryo.

Seasonal Associated Increases in Embryonic Mortality in Dairy Cattle

Seasonal exposure of dairy cows to elevated ambient temperatures is associated with decreased first service conception rates and increased number of services per conception (Poston et al., 1962; Gwazdauskas et al., 1973; Rosenburg et al., 1977; Cavestany et al., 1985). Similarly, experimental application of heat stress causes increased embryonic mortality and reduced development (Putney et al., 1988a; 1989a; Ealy et al., 1993). As a result, cows bred during the summer months in hot climates remain open for a longer period of time than cows bred at other times during the year (Fuquay, 1981).

Negative effects of elevated ambient temperature on fertility are closely associated with increases in body temperature (Gwazdauskas et al., 1973). Moreover, rectal temperatures are similar to those in utero and thus provide an accurate assessment of the degree of hyperthermia that may be experienced by an animal. Ulberg and Burfening (1967) demonstrated that pregnancy rate decreased with each degree of increase in rectal temperature. In hyperthermic dairy animals rectal temperatures may exceed 41°C (Putney et al., 1988a; 1989a; Wolfenson et al., 1993).

There are several lines of evidence that effects of heat stress are more prominent when occurring at or near the time of estrus (Fallon, 1962; Long et al., 1969; Putney et al., 1989a). Heat stress administered for a 10 h period beginning at the onset of estrus increased the incidence of retarded and/or abnormal embryos with degenerated blastomeres (Putney et al., 1989a). Decreases in environmental temperatures of any magnitude for 1 to 3 days before or after day of breeding, when maximum temperatures on the day of breeding were $\geq$ 27°C, resulted in higher pregnancy rates (Cavestany et al., 1985). Cooling dairy cows to basal body temperatures in a climatically controlled barn before insemination and for 1 to 6.5 d following artificial insemination increased pregnancy rates (Stott & Wiersma, 1976). Similar findings were reported by Ealy et al. (1994). Taken together, these observations suggest that the maturing oocyte or early embryo is very susceptible to elevated temperatures.

Deleterious effects of heat stress also occur very early in pregnancy. Monty and Racowsky (1987) reported an increase in the percentage of degenerate embryos recovered on d 7 of pregnancy from superovulated dairy cows between the months of June and September compared to those recovered between October and May. Also,

exposure of superovulated dairy heifers to 42°C in climatic chambers from 30 h post estrus until d 7 of pregnancy elevated rectal temperature to $\geq$ 41°C and reduced the percentage of normal embryos (51.5% for control versus 20.7% for heat stressed heifers; Putney et al., 1988a). Moreover, stressed heifers had a higher incidence of abnormal and retarded embryos with degenerate blastomeres.

Embryos are most susceptible to elevated temperature during the early cleavage stages. Heat stress administered on d 1 of pregnancy to superovulated dairy cows reduced embryonic viability and development (Ealy et al., 1993). Maternal heat stress administered on other days of pregnancy (d 3, 5 and 7) were minimal, suggesting that embryos acquire the ability to withstand elevated temperatures as they proceed in development. In agreement, Putney et al. (1989c) demonstrated that pregnancy rates of lactating cows on d 21 during summer heat stress conditions were higher following transfer of d 7 morula or blastocysts to recipient animals (48%) as compared to cows bred via artificial insemination (18%). As compared to heat stress at d 0 to 1, heat stress in other species is less effective at decreasing embryonic survival by d 3 to 5 in the ewe (Dutt, 1963) and by d 5 in the pig (Tompkins et al., 1967).

Indirect Effects of Elevated Temperature on Embryonic Survival

Data collected in sheep (Alliston & Ulberg, 1961) suggest that heat stress compromises function of both the maternal environment and the early embryo and that direct effects of heat stress on the embryo are more detrimental to fertility. Pregnancy rates for embryos transferred from a thermoneutral donor to nonstressed recipients were 56.5%. In contrast, pregnancy rates of embryos transferred on d 3 after breeding from

heat stressed donors to a nonstressed recipient averaged 9.5%; pregnancy rates of embryos transferred from a nonstressed donor to a heat stressed recipient were 24%.

One could postulate two mechanisms by which heat stress could compromise the oviductal and uterine environment; direct effects of elevated temperature on function of the reproductive tract and alterations in maternal hormonal secretion or blood flow that would compromise reproductive tract function. There is evidence for both types of effects. Protein synthesis and secretion in the oviduct and uterus can be altered by heat shock. Malayer and Hansen (1990) reported that heat shock increased secretion of nondialyzable ^{3}H-labeled macromolecules by both oviducts of Brahmans but depressed secretion by the oviduct ipsilateral to the side of ovulation in Holsteins. Exposure of Hereford cows to heat stress between d 8 and 17 of the estrous cycle increased protein content in uterine luminal flushes (Geisert et al., 1988). Similarly, culture of endometrial explants at 43°C increased both protein synthesis and secretion by the endometrium (Malayer et al., 1988; Malayer and Hansen, 1990). In contrast, other studies have reported decreased protein synthesis (Geisert et al., 1988) or no effect (Putney et al., 1988b) when these tissues are exposed to elevated temperatures.

Heat stress also causes increased synthesis of the eicosanoid prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in vivo and this effect can be minimized in vitro by exposing cultured endometrium to elevated temperature. Geisert et al. (1988) demonstrated that content of $PGF_{2\alpha}$ in lumenal flushes tended to be higher in the ipsilateral uterine horn of heat-stressed cows when a conceptus was present. Moreover, Wolfenson et al. (1993) demonstrated that oxytocin-induced changes in plasma concentrations of 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) tended to be higher in heat stressed heifers at d 17 of pregnancy

when compared to those maintained at thermoneutral temperature. Similar effects have been reported for cultured endometrium. Exposure of endometrial tissues to 42°C increased the secretion of $PGF_{2\alpha}$ (Putney et al., 1988b; 1989b; Malayer et al., 1990). Secretion of $PGF_{2\alpha}$ in response to an oxytocin challenge is minimal from perfused endometrium from d 17 pregnant cows when cultured at 39°C (Putney et al., 1989b). However, exposure to heat shock increased responsiveness of endometrial perfusions from pregnant cows to oxytocin. Collectively, these observations suggest that the ability of the conceptus to inhibit $PGF_{2\alpha}$ synthesis may be compromised when cows are exposed to heat stress (Putney et al., 1988c). Also, effects of heat shock on uterine $PGF_{2\alpha}$ may be exacerbated because of corresponding changes in conceptus synthesis and secretion of interferon-τ (IFN-τ), a protein required for extension of the life of the corpus luteum (Thatcher et al., 1989). Culture of conceptuses at 43°C reduced (Putney et al., 1988c) or increased (Geisert et al., 1988) synthesis and secretion of IFN-τ.

Heat-induced alterations in oviductal and uterine function may in part be due to altered uterine blood flow or secretion of ovarian steroids such as estrogen and progesterone. Uterine blood flow is reduced in cattle (Roman-Ponce et al., 1978) and sheep (Alexander et al., 1987) following exposure to heat stress. Roman-Ponce et al. (1978) demonstrated that uterine blood flow induced by an exogenous dose of estradiol was less for those cows exposed to the sun without access to shade.

Circulating concentrations of progesterone have been reported to be reduced (Rosenburg et al., 1977; Wolfenson et al., 1988; Younas et al., 1993; Howell et al., 1994), elevated (Abilay et al., 1975; Vaught et al., 1977; Roman-Ponce et al., 1981; Collier et al., 1982) or unaffected (Gwazdauskas et al., 1973; Wise et al., 1988; Badinga

et al., 1993) by heat stress. Similar findings have been reported for estradiol (Christison & Johnson, 1972; Folman et al., 1983; Gwazdauskas et al., 1981; Wise et al., 1988). Noted effects on altered synthesis of gonadal steroids are reported to be dependent (Madan & Johnson, 1973; Vaught et al., 1977; Roman-Ponce et al., 1981; Wise et al., 1988) or independent (Vaught et al., 1977; Gwazdauskas et al., 1981; Younas et al., 1993) of heat-induced changes in circulating gonadotropins. Disparity of results may be related to a number of factors. In particular, Gilad et al. (1993) showed that heat-induced alterations in gonadotropins are dependent upon concentrations of estradiol in plasma. In cows with low circulating estradiol, mean basal concentrations of LH and FSH as well as the GnRH-induced FSH and LH release were lower in heat stressed cows compared to cooled.

An effect of heat stress on FSH and LH release could be important for fertility because of the described role for gonadotropins dictating follicle selection, function and turnover during the estrous cycle (Lucy et al., 1992). Increased pulse frequency of LH prevented follicle turnover by maintaining estradiol secretion and growth of the dominant follicle originating during the first wave of follicle growth (Savio et al., 1990). Pregnancy rates resulting from the ovulation of such follicles are lower compared to cows ovulating a newly recruited follicle (Savio et al., 1993).

Effects of heat stress on the functional and structural integrity of the oocyte within the follicle remain to be determined. However, exposure of lactating Holsteins to chronic effects of heat stress during the summer alters follicular growth (Smith, 1992; Badinga et al., 1993). Heat stress increased the number of small follicles (Smith, 1992),

decreased the size of the dominant follicle and increased the number of subordinate follicles (Smith, 1992; Badinga et al., 1993) present on the ovary.

Direct Effects of Elevated Temperatures on Embryonic Survival

Deleterious direct effects of elevated temperatures on cells may occur as a consequence of damage to proteins, membranes, or DNA as well as induction of apoptosis. Much evidence exists to demonstrate that heat shock can decrease embryonic survival but specific lesions in cellular function are mostly unknown. The following is a review of the direct effects of elevated temperature on a variety of cell types including gametes and the early embryo.

Effects of Heat Shock on Proteins, DNA, and Membrane Integrity

Exposure of cells to elevated temperatures increases the kinetic energy of all cellular molecules; as a consequence, thermal denaturation and aggregation of proteins may occur (Kampinga et al., 1995). Denatured proteins can be found throughout the cell following heat shock, particularly in the nuclear, microsomal and mitochondrial fractions (Lepock et al., 1993). Also, exposure of cells to elevated temperatures causes disassembly of microtubules (Wiegant et al., 1987), intermediate filaments and microfilaments (Welch & Suhan, 1985). Conformational changes in membrane associated proteins also have been reported (Lepock et al., 1983). Kampinga et al. (1995) proposed that a threshold of damage exists in cells and that a number of sets of critical proteins need to be damaged before this threshold is exceeded and the cell dies. Protein synthetic capability of the cell is also compromised and will be described in detail in Section II of this review.

Hyperthermic killing of cells may also be related to alterations in the integrity of DNA. Exposure of cells to temperatures $\geq 45°C$ induced unwinding of supercoiled DNA, breaks in double stranded DNA and chromosomal exchanges (Vig, 1979; Sherwood et al., 1987; Mackey et al., 1988). Increased DNA damage could in part be a result of free radical production (see next section) as well as decreased activity of topoisomerase enzymes involved in DNA repair (Dewey & Esch, 1982).

Heat shock also alters membrane integrity of the cell. Exposure of murine oocytes to 43 °C for 30 min resulted in hyperthermic killing by increasing the permeability of the oolema (Hendrey & Kola, 1991). This effect could be due to expansion of the lipid bilayer and increased formation of inverted micelles that has been reported in other cell types (Overath et al., 1970; McElhaney, 1974; Yatvin, 1977). Also, heat shock increases calcium influxes in cells exposed to elevated temperatures (Stevenson et al., 1987). Theoretically, severe alterations in calcium influxes could result in cell death via apoptosis (Schwartzman & Cidlowski, 1993).

Free Radicals

Most of the molecular oxygen consumed by cells is reduced to H_3O; approximately 10%, however, is only partially reduced so that highly reactive oxygen species are formed (Allen, 1991). These noxious molecules have been implicated as mediators of heat-induced killing of cells (reviewed by Loven, 1988) because exposure of cells to elevated temperature increases oxygen consumption (Leenders & Berendes, 1972), degradative products of lipid peroxidation (Skibba et al., 1986) and antioxidant enzymes (Loven et al., 1985; Kondo et al., 1993), decreases pool of antioxidants (Shrieve et al., 1986; Aréchiga et al., 1995) and cell survival (Mitchell et al., 1983; Ealy et al.,

1992; Arechiga et al., 1995). Moreover, deleterious effects of heat shock are partially ameliorated upon addition of extracellular antioxidants (Ealy et al., 1992; Malayer & Hansen, 1992; Arechiga et al., 1995; see section IV). Finally, depletion of antioxidants increases thermal sensitivity of cells (Mitchell et al., 1983; Harris et al., 1991).

The most abundant free radical is $\cdot O_2$ (superoxide; reviewed by Loven, 1988). Within the cell, $\cdot O_2$ spontaneously dismutes to hydrogen peroxide (H_2O_2) or may remove electrons from various cellular components such as lipids or sulfhydryl groups (Allen, 1991). In addition, H_2O_2 and $\cdot O_2$ can react with metals to form highly reactive $\cdot OH$ (Halliwell & Gutteridge, 1984). Resulting products are highly reactive. Deleterious effects of $\cdot O_2$ and H_2O_2 are in part related to an ability of these molecules to initiate lipid peroxidation (Wefers and Sies, 1983) thereby altering membrane fluidity (Loven, 1988), inactivate cellular enzymes (Halliwell & Gutteridge, 1984) and damage DNA (Abe et al., 1995) within the cell.

Oocyte

Alterations in nuclear maturation and protein synthetic capability may account in part for deleterious effects on subsequent development of the oocyte following fertilization. Baumgartner and Chrisman (1987) reported that meiotic maturation was disrupted in 40% of the analyzable ova obtained from mice that were heat stressed during the first 12.5 h of maturation. Oocytes that were heat stressed stopped maturing at diakinesis-metaphase-I in 4% of the oocytes analyzed and retention of the polar body chromosomes was observed in 2%. Remaining oocytes had atypical morphologies including dispersed chromatin, micronuclei or degenerating chromatin. Heat stress during the later stages of maturation disrupted spindle formation and reduced ability of

the mouse oocyte to progress to metaphase II (Baumgartner & Chrisman, 1981a; 1981b). Similar findings in the bovine also indicate that elevated temperatures may directly compromise the function of the oocyte. Lenz and coworkers (1983) demonstrated that culture of bovine oocytes for 24 h at 41°C reduced the frequency of oocytes that progressed to metaphase II. Other effects of heat shock on the mouse oocyte during maturation include alterations in membrane integrity (Hendrey & Kola, 1991), increased fragmentation, cleavage of large polarcytes and polarcyte longevity, alterations in chromosome number and nuclear conformation (Baumgartner & Chrisman, 1981a; 1981b) and decreased intracellular protein synthesis (Curci et al., 1987). The latter effect of heat shock is of importance because ability of the oocyte to mature at both the nuclear and cytoplasmic level is dependent upon the synthesis of critical proteins such as the cyclin dependent protein known as maturation promoting factor (Hashimoto & Kishimoto, 1988; Nurse, 1990).

Effects of elevated temperature are in part due to heat-induced alterations in the maternal environment. Woody and Ulberg (1964) demonstrated that effects of heat stress were minimal in the ewe when oocytes were transferred to a maternal environment that had not been previously exposed to elevated temperature. In this experiment, ewes were exposed to an elevated ambient temperature of 32°C just prior to ovulation. Shortly afterwards, oocytes were recovered and placed in oviducts of control ewes containing spermatozoa. Pregnancy rates resulting from ova that had been heat stressed or maintained at homeothermic temperatures were similar (45 versus 35%, respectively).

Elevated temperatures also compromise the function of the companion cumulus cells surrounding the oocyte. Lenz et al. (1983) demonstrated that incorporation of

radiolabeled glucosamine into hyaluronic acid was significantly reduced by culturing cumulus cells at 41°C for 24 h. Expansion of cumulus via FSH-induced production of hyaluronic acid is required for the oocyte to continue the maturation process (Ball et al., 1980). Additionally, cumulus cells supply the oocyte with nutrients, metabolites and possibly low molecular weight signals via gap junctions (Lawrence et al., 1978). More recently a protective role of cumulus cells has been described. Deleterious effects of high oxygen concentrations on continued development of the oocyte following fertilization were ameliorated partially by the presence of multiple layers of cumulus cells (Eppig & Wigglesworth, 1995).

Sperm

Like females, bulls experience periods of transient infertility during periods of heat stress (Erb et al., 1942). Hyperthermia decreased sperm motility and concentration in ejaculates (Casady, 1953; Johnson & Branton, 1953). Effects of heat stress occur very early in development with early spermatids and spermatogonia being the most sensitive (DeAlba & Riera, 1966; Amann & Schonbacher, 1983). Artificial insemination using frozen semen has largely removed infertility attributable to the male during the summer due to alterations in semen quality in the United States. Even so, evidence indicates that mature sperm could be susceptible to elevated temperature in the reproductive tract of the hyperthermic female. Exposure of rabbit sperm to elevated temperature either in vitro (Burfening & Ulberg, 1968) or in vivo (Howarth et al., 1965; Bellve, 1973) reduced embryonic development without affecting fertilization or cleavage rates of resulting embryos. Monterroso et al. (1995) reported that exposure of frozen/thawed sperm to 41 or 42°C for 3 h slightly reduced viability and decreased velocity of sperm, as determined

using a Hamilton Thorn Motility Analyzer, without altering cleavage rates of oocytes following fertilization. Exposure of spermatozoa to a more severe heat shock (43°C) decreased the proportion undergoing swim-up.

Early Embryo

Deleterious effects of heat stress during preimplantation development on reducing embryonic survival have been well documented in cattle (Putney et al., 1988a; Ealy et al., 1993; Monty & Racowsky, 1987), sheep (Dutt, 1963), swine (Tompkins et al., 1967; Wetteman et al., 1988), mice (Bellve, 1976) and rabbits (Wolfenson & Blum, 1988). These data suggest that the early embryo is very susceptible to the effects of elevated body temperature. Exposure to heat stress on d 1 (Ealy et al., 1993) or d 1-7 (Monty & Racowsky, 1987; Putney et al., 1988a) of pregnancy in cattle decreased the number of blastocysts and increased the number of embryos that are morphologically abnormal and retarded in development when recovered from superovulated animals. In mice, exposure of females to elevated ambient temperatures following mating increased embryonic mortality caused primarily by an arrest in development at the 2-cell stage (Bellve, 1973).

Individual embryos may vary in their sensitivity to elevated temperatures. Bellve (1976) reported that 20% of 2-cell embryos were affected only partially by exposure to maternal hyperthermia because one blastomere is able to undergo continued cleavage divisions. Such an effect could account for an accumulation of embryos containing 3, 5 or 9 blastomeres following exposure to elevated temperatures (Ulberg & Sheean, 1973). Continued but retarded cleavage of both blastomeres is also a possibility (Bellve, 1976).

Using culture systems, it is clear that part of the deleterious effects of heat stress may be due to direct effects of elevated temperature on the early embryo. Alliston et al.

(1965) demonstrated that culture of 1-cell fertilized rabbit ova at 40°C for 6 h increased embryonic mortality following transfer to synchronous pseudopregnant females. Moreover, embryos were more susceptible to elevated temperature during the first cell division than during the second (Alliston et al., 1965). In this particular experiment, the survival rate of embryos cultured at 40°C after transfer to a nonstressed doe was lower for embryos heat shocked during the first cell division than for embryos heat shocked during the second cleavage division. Similar findings were observed for mouse embryos by Gwazdauskas et al. (1992): 1-cell mouse embryos were more sensitive to heat shock than 2-cell embryos. Developmental changes in thermal sensitivity have also been reported for bovine embryos (Ealy et al., 1995). Culture of IVF-derived 2-cell embryos at temperatures $\geq$ 41°C reduced the number of embryos that were able to continue in development. In contrast, effects of elevated culture temperatures were minimal by the morula stage. Cumulatively these data suggest that embryos become more resistant to heat damage as the number of cells increase. See sections 3 and 4 in this review for possible biochemical mechanisms responsible for developmental acquisition of thermotolerance.

Specific mechanisms responsible for heat-induced killing of early embryos remain undefined but may be related to alterations in RNA and protein synthesis. In support of this, retarded development in mouse embryos in response to elevated temperature is coincident with fewer blastomeres of 4- and 8-cell embryos, reduced ability to incorporate [^{3}H]uridine (Bellve, 1972; 1976) and an overall decrease in the protein synthetic capability of the early embryo (Hahnel et al., 1986). Bellve (1976) demonstrated that not all blastomeres of an effected embryo failed to incorporate

[^{3}H]uridine. In fact, some blastomeres of heat shocked embryos exhibited a distribution pattern of isotope similar to that of blastomeres obtained from normal embryos at the same chronological stage of development. In contrast, arrested blastomeres incorporated very little [^{3}H]uridine; when detectable, the isotope was confined to the nucleus and never assumed the pattern typical of blastomeres at later stages of development. This observation suggested that some blastomeres are able to remain viable for longer periods of time following stress than others. Effects of heat shock on the overall protein synthetic capability of the early embryo also has been noted. In particular, exposure of preimplantation murine embryos to 43°C for 1 h was sufficient to reduce the synthesis of intracellular proteins by 75-85% at all stages examined (Hahnel et al., 1986). Similar findings were noted using rabbit embryos (Heikkila & Schultz, 1984).

Direct effects of elevated temperature on the conceptus have been noted during later stages of development. Culture of bovine conceptuses obtained on d 17 of pregnancy at 43°C for 18 h reduced incorporation of [^{3}H]leucine into intracellular and secreted proteins by conceptuses and reduced the secretion of IFN-τ by 75% (Putney et al., 1988c). In contrast, exposure of conceptuses to heat stress in utero reduced conceptus weights and increased the synthesis and release of IFN-τ following 24 h of culture (Geisert et al., 1988). Deleterious effects of elevated temperature on conceptus development have also been seen using milder temperatures; culture of pig embryos ranging from 12.5 to 13 days of age at 41°C retarded subsequent development and increased the frequencies of abnormalities including microcephaly, failure of somite formation and unclosed neural folds (Trujano & Wrathall, 1985). Similar effects have been noted for postimplantation rat embryos (Mirkes & Cornel, 1992).

Heat Shock Response

The environment in which cells reside is rarely static. For continued survival, cells have evolved homeostatic adaptive mechanisms that allow for a rapid response to a hostile environment. The molecular characteristics of the response to heat shock is highly conserved among all organisms studied and includes a change in the pattern of gene expression. This phenomenon was first characterized in Drosophila. Exposure of cells to heat shock induced a puffing pattern on polytene salivary gland chromosomes (Rotissa, 1962) and synthesis of a distinct set of proteins referred to as heat shock proteins (Tissieres et al., 1974). Thus, the phrase "heat shock response" was readily adopted. The requirement of such a response following heat shock was quickly established because transcriptional or translational inhibitors abolished heat-induced production of heat shock proteins and consequently decreased cell survival (Pardue et al., 1992). The heat shock response is dependent upon the severity and duration of elevated temperature and is fully reversible because protein synthesis is restored to prestress patterns when cells are returned to homeothermic temperatures (Lindquist, 1986; Lindquist & Craig, 1988; Welch, 1993).

Additional studies designed to answer the question of how cells cope with changes in their environment revealed that a variety of stressors including amino acid analogs (Kelley & Schlesinger, 1978), anoxia (Sciandra et al., 1984), ethanol (Li & Werb, 1982), serum or mitogen supplementation (Wu & Morimoto, 1985; Wu et al., 1986), glucose deprivation (Sciandra & Subjeck, 1983), high pH (Whelan & Hightower, 1985), oxidants (Kim et al., 1983; Spitz et al., 1987) and bacterial and viral infections

(Rose & Khandjiam, 1985) also elicited synthesis of heat shock proteins. Thus, the adaptive response is referred to as the stress response rather than heat shock response.

It is now known that heat shock proteins are critical for normal cellular homeostasis. Many of the stress-inducible proteins have cellular counterparts that are constitutively expressed and act as molecular chaperones within the cell (Hendrick & Hartl, 1993). Their major importance lies in their ability to aid in protein folding, targeting and trafficking. For this section a review of the current literature detailing the role of heat shock proteins and factors involved in heat-induced regulation of transcription and translation of heat shock proteins will be discussed.

Heat Shock Proteins

Six families of heat shock proteins have been characterized. These proteins are identified according to molecular weight and include HSP110, HSP90, HSP70, HSP60, HSP27 and ubiquitin (Kochevar et al., 1991; Nover & Scharf, 1991). Within each family, proteins are highly conserved across species and retain similar functions. For example, bacterial and human heat shock protein genes share approximately 40% homology with one another (Craig, 1985; Lindquist, 1986). While heat shock proteins were thought initially to function only in the injured cell, it is now known that they are constitutively expressed and play a crucial role as molecular chaperones in the absence of stress. Hendrick and Hartl (1993) defined molecular chaperones as proteins that stabilize an otherwise unstable conformer of another protein. Further, by controlled binding and release of the substrate protein, chaperones assist proteins in folding, oligomeric assembly and transport to subcellular compartments within the cell. The following

section is a review of the role of heat shock proteins in cellular homeostasis and in thermotolerance during periods of heat shock.

Induced Thermotolerance

Induction of thermotolerance (a phenomenon whereby prior exposure of cells to heat shock confers resistance to a subsequent more severe heat shock) occurs in murine and bovine embryos (Ealy & Hansen, 1994). In this study, prior exposure of embryos to 42°C made embryos resistant to a more severe heat shock (43°C for 2 h) at the 8-cell stage if embryos were developed in vitro and the blastocyst stage if developed in utero. Many other cells have been shown to be capable of induced thermotolerance (Nover & Scharf, 1991). Correlative evidence suggests that heat-induced synthesis of heat shock proteins are required for thermotolerance. Studies using pulse incorporation of [^{35}S]methionine following a conditioning treatment of 43°C for 30 min demonstrated that thermotolerance in Morris hepatoma cells was accompanied by elevated synthesis of many heat shock proteins (Landry et al., 1982). Development of thermotolerance (as assessed by the number of cells surviving a second treatment of 43°C for 2.5 h) and induction of heat shock protein synthesis were complete following a 6 to 8 h period. Resistance to elevated temperature disappeared between 60 to 80 h following exposure to 43°C. Similar findings were reported by Li and Werb (1982). Moreover, exposure of Chinese hamster ovary fibroblasts to heat shock (41-46°C) greatly enhanced the synthesis of heat shock proteins compared to nonheated cells and increased cell survival by 100 to 1,000,000 fold when cells were challenged by a subsequent otherwise lethal heat shock. Lee and Dewey (1987) demonstrated that inhibition of protein synthesis

using cycloheximide and puromycin was sufficient to ablate any beneficial effects of prior exposure of Chinese hamster ovary cells to a sublethal heat shock.

HSP70

To date, HSP70 is the most studied group of heat shock proteins. In *Drosophila*, HSP70 is the product of a single gene (Lindquist and Craig, 1988). In contrast, up to 10 genes in humans (Mues et al., 1986), 6 genes in mice (Lowe & Moran, 1986) and 4 genes in rats (Lisowska et al., 1994) have been identified. Members of this particular family may be divided into two groups: 1) cognate proteins, which are present under optimal growth conditions and 2) those that are stress-induced. Homologs of the cognate proteins are found throughout the cytoplasm, nucleus, endoplasmic reticulum and mitochondria of the cell (Hendrick & Hartl, 1993). In contrast, heat-induced proteins are predominantly found in the nucleus during and immediately following stress (Pelham, 1984; Riabowol et al., 1988; Hayashi et al., 1991; Li et al., 1991).

Heat shock cognate proteins (HSC70) range in molecular weight from 70 to 76 kDa with a pI of 5.3-5.6 (Dworniczak & Mirault, 1987; Sorger & Pelham, 1987; Giebel et al., 1988; Masumi et al., 1990). A variety of regulatory sequences are found within the promoter region of these genes indicating that factors other than heat shock regulate their expression (Sorger & Pelham, 1987). It is not surprising, then, that heat shock enhances the synthesis of these proteins only 2- to 3-fold (Milarski et al., 1989).

Heat-inducible forms of HSP70 are slightly lower in molecular weight (68 to 74 kDa) and more basic (pI ranges from 5.6 to 6.3) than the constitutively synthesized HSC70 proteins; heat shock causes a 20- to 30-fold increase in synthesis (Lowe & Moran, 1984; Milarski et al., 1989; Hunt & Calderwood, 1990; Masumi et al., 1990).

The inducible form of HSP70 is either absent in cells cultured at homeothermic temperatures or present in very low amounts: it will be identified as HSP68 in this dissertation. The term HSP70 will be used to refer to the HSP70 family as a class of proteins. A third member of the HSP70 group called glucose regulatory protein (GRP78) is nonresponsive to heat shock but dependent upon glucose for regulation of its synthesis (Shiu et al., 1977; Kochevar et al., 1991).

Proteins within the HSP70 family share a high degree of sequence homology with one another. Heteroduplex formation using different stringencies revealed that the hsp70 gene is 74% identical for yeast and *Drosophila* and 85% for mouse and *Drosophila* (Moran et al., 1983). Moreover, the major heat-inducible rat hsp70 gene is 98% identical to mouse hsp70 (Lisowska et al., 1994) and only differs at 3 positions when compared to the bovine hsc70 gene (DeLuca-Flaherty & McKay, 1990). Grosz et al. (1992) showed a syntenic conservation of hsp70 genes in cattle and humans. HSP70 differs from *E. Coli* and *Drosophila* by 55% (Dworniczak & Mirault, 1987).

Specific regions of homology within HSP70 have been identified (reviewed by Craig et al., 1993; Feige & Polla, 1994; Hendrick & Hartl, 1995). Using partial proteolytic digestion, Chappell et al. (1987) demonstrated that HSP70 is characterized by the presence of a 45 kDa ATPase domain located in the amino-terminal region. The crystal structure of bovine HSC70 isolated from brain tissue indicated that the ATPase domain consists of an identical fold to two ATPases (Flaherty et al., 1991). This region shares a high structural homology with ATPase domains of hexokinase (Flaherty et al., 1990) and actin (Flaherty et al., 1991). Binding of ATP invokes a conformational change that can be transmitted to the carboxy-terminus (Liberek et al., 1991).

The carboxy-terminal region of HSP70 is characterized by two distinct regions: an 18 kDa peptide binding site and a more variable region of ~10 kDa (Chappell et al., 1987; Wang et al., 1993; Hendrick & Hartl, 1995). The peptide binding domain was initially modelled on the structure of MHC class I antigen (Flajnik et al., 1991; Feige & Polla, 1994); localization is within the final 150 amino acids of the carboxy-terminal region (Sorger & Pelham, 1987). HSP70 associates with hydrophobic amino acids (Flynn et al., 1991; Blond-Elguindi et al., 1993) via a unique secondary topology (Morshauser et al., 1995). The functional significance of the more variable 10 kDa region remains to be determined. Additionally, both the amino and carboxy-terminal regions of HSP70 contain signal sequences for targeting to specific organelles within the cell (Hendrick & Hartl, 1995).

Binding of HSP70 with cellular peptides is dependent upon ATP (Flynn et al., 1991; Craig et al., 1993; Todd et al., 1994; Hendrick & Hartl, 1995). When HSP70 is bound to ATP, peptides are released more rapidly than when HSP70 is bound to ADP (Palleros et al., 1994; Schmid et al., 1994). Peptide binding and other cellular cofactors (Echols, 1990; Georgopoulos, 1992) stimulate hydrolysis of ATP which in turn allows for dissociation of peptides from HSP70 (Flaherty et al., 1991). Beckman et al. (1990) demonstrated that fewer proteins coprecipitated when pulse chased labeled cell lysates were incubated with exogenous ATP.

The role and functional significance of HSP70 interacting with cellular proteins is dependent upon the location of HSP70 within the cell and is related to an ability to fold/unfold nascent polypeptides and renature malfolded proteins. For example, in the cytosol, interaction of HSP70 with newly synthesized proteins appears to occur

cotranslationally, because nascent polypeptides released prematurely from polysomes in vivo were immunologically isolated in a complex with HSP70 (Beckman et al., 1990). HSP70 also renatures malfolded proteins that might occur as a result of normal cellular metabolism. Using a rabbit reticulocyte lysate system, Schumacher et al. (1994) demonstrated that renaturation of a denatured luciferase protein was highly correlated with levels of HSP70. Moreover, addition of purified HSP70 along with an ATP-regenerating system renatured luciferase activity to greater than 20% of its original activity.

Of the various intracellular proteins that are synthesized within the cytosol, many are destined ultimately to reside in organelles such as the endoplasmic reticulum, mitochondria and the nucleus. Mechanisms required for translocation of such proteins have been best studied in yeast and are dependent upon HSC70, ATP and other cytosolic factors (Hendrick & Hartl, 1993). Addition of HSP70 along with other cytosolic factors stimulated translocation of proteins into the endoplasmic reticulum and mitochondria (Chirico et al., 1988; Murakami et al., 1988; Craig et al., 1993). In contrast, depletion of HSP70 in yeast cells promotes accumulation of specific precursors for mitochondrial and secreted proteins (Deshaies et al., 1988; Hendrick & Hartl, 1993). Interestingly, denaturation of proteins bypasses the requirement of HSP70 for translocation indicating that a major function of HSP70 is to unfold proteins prior to translocation (Chirico et al., 1988; Craig et al., 1993)

HSP70 also associates with a variety of steroid hormone receptors (Kost et al., 1989; Pratt et al., 1992). Following ligand binding, HSP70 dissociates in an ATP-dependent manner (Kost et al., 1989; Onate et al., 1991; Smith & Toft, 1993). In this

system, HSP70 may be required for binding of other associated proteins to the steroid receptor complex. Addition of anti-HSP70 monoclonal antibody to reticulocyte lysates inhibited HSP90 from binding to the complex (Smith & Toft, 1993).

Cell cycle variations in HSP70 have been reported by Milarski and Morimoto (1986). This phenomenon was later studied using flow cytometry by dual labeling HeLa cells with propidium iodide and antibodies specific for HSP70 (Hang et al., 1995). Amounts of HSP70 increased approximately 30% from the G1 to S phase and by 65% during the G2/M phase. Neither mitotic selection nor serum stimulation induced higher levels of HSP70. Functions of HSP70 during this time remain unknown but may be associated with DNA replication and rearrangement of the cytoskeleton. Using indirect immunofluorescence, Brown et al. (1996a) demonstrated that HSP70 is localized within the centrosome during interphase and mitosis.

A role for HSP70 in targeting intracellular proteins for degradation has also been described. Chiang et al. (1989) immunoprecipitated a 73 kDa protein that is associated with the region of a peptide that targets intracellular proteins for lysosomal degradation. Intracellular concentrations of HSP70 was increased by serum withdrawal and in the presence of ATP enhanced protein degradation in the form of lysosomal proteolysis. HSP70 is also a critical component of the endosomal pathway utilizing clatherin coated pits for endocytosis of transmembrane receptors. Following endocytosis, HSP70 disassembles the clathrin lattice by associating with a glycine and proline enriched region of one of the two light chain regions of clathrin (DeLuca-Flaherty et al. 1990) allowing vesicles to enter the endosomal pathway.

HSP70 and Thermotolerance

Of all the heat shock proteins induced by heat shock, HSP70 is the predominant protein in heat shocked cells (DiDomenico et al., 1982). Induced thermotolerance in HeLa cells is highly correlated with expression and accumulation of HSP70; magnitude of synthesis of these proteins is a function of the severity of heat shock (Mizzen & Welch, 1988). Interestingly, those cells that were made thermotolerant via prior exposure to 43°C and then challenged with 45°C produced significantly less HSC72/73 than did nontolerant cells exposed to the same treatment.

More direct evidence for involvement of HSP70 in cellular thermotolerance comes from studies that utilize methods to determine effects of underexpression and overexpression of HSP70. Riabowol et al. (1988) demonstrated that microinjection of an HSP70 antibody into fibroblasts impaired heat-induced translocation of HSP70 into the nucleus. Moreover, injected cells were unable to survive following a brief incubation at 45°C. Similarly, overexpression of the hsp70 gene that was inserted into a plasmid containing the eukaryotic gene for dihydrofolate reductase reduced expression of the endogenous gene by 90% in modified cells and increased thermosensitivity (Johnston & Kucey, 1988). In contrast, overexpression of HSP70 in rat fibroblasts via transfection of an hsp70 gene conferred heat resistance. Higher levels of expression were associated with increased resistance to 45°C. Similarly, microinjection of HSP70 mRNA into murine oocytes increased thermal resistance of oocytes to an otherwise lethal heat shock of 43°C (Hendrey & Kola, 1991).

Expression of HSP68 alone may not be sufficient to confer thermotolerance in all cells (Easton et al., 1987). Thermotolerance was induced in Chinese hamster V79 cells

without any noticeable increases in the synthesis of HSP68 (Haytayama et al., 1991). Moreover, variation in development of cross-tolerance in postimplantation mouse embryos from two different strains (BALB/c and SWV) was not correlated with differences in heat-induced synthesis of HSP68 (Kapron-Bras & Hales, 1992). In both strains, there was a rapid increase HSP68 following exposure to 43°C but no excess in the SWV strain that developed thermotolerance. In fact, amounts of HSP68 were less in the thermotolerant strain. Differences in other heat shock proteins are unlikely because heating conditions that induced thermotolerance in human epidermal keratinocytes stimulated the production of a variety of heat shock proteins (Maytin et al., 1990) without correlating with thermotolerance. Large increases in heat shock proteins were observed in cells that did not have good survival.

Instead thermotolerance may be dependent upon the synthesis of a novel cytoplasmic RNA (Fung et al., 1995). In this study, a strain of *Tetrahymena thermophila* was genetically constructed that was devoid of a small cytoplasmic RNA referred to as G8. Exposure of cells to heat shock increased synthesis of HSP70 and other heat shock proteins but did not become thermotolerant when exposed to a mild heat shock.

Increased denaturation and aggregation of ribosomal components occur within the nucleolus during heat shock (Welch & Suhan, 1986). Changes in nuclear morphology and assembly and export of ribosomal material is blocked for several hours (Pelham, 1982). Under nonstressed conditions, HSP70 is found almost exclusively in the cytoplasm. Within minutes following heat shock (41-45°C), indirect immunofluorescence reveals that HSP70 is transported and concentrated in the nucleoli of the cell (Pelham, 1984; Riabowol et al., 1988; Hayashi et al., 1991; Li et al., 1991).

Pelham (1982) suggested that the rapid transport of HSP70 to the nucleolus following heat shock aids in nucleolar recovery by reassembling damaged ribosomal components and other ribonuclear peptides. Transfection of COS cells from *Drosophila* with hsp70 prior to heat shock allowed for a more rapid recovery of nucleolar morphology when compared to nontransfected cells.

Also associated with heat shock is alterations in the translational machinery. In HeLa cells, translation can be increased following exposure to a mild heat shock (Mizzen & Welch, 1988). It remains to be determined whether or not this effect is due to interactions of specific heat shock proteins with translational machinery. HSP70 can be immunolocalized to the centrosome during interphase and mitosis (Brown et al., 1996a). Following heat shock, the centrosome could not be identified and cells were unable to support regrowth of microtubules (Brown et al., 1996b). However, during recovery a strong correlation between regeneration of the centrosome and HSP70 was noted. Microinjection of HSP70 antibody blocked centrosomal reassembly and outgrowth of microtubules following heat shock. In contrast, microinjection of purified HSP70 accelerated both repair and function of the organelle. Other beneficial effects of HSP70 following heat shock may be due to their ability to associate with denatured proteins (discussed in a following section).

HSP110

Unlike most other heat shock proteins, HSP110 is localized in the nucleolus of nonshocked and heat shocked cells (Lindquist & Craig, 1988). Following prolonged exposure to heat shock, microscopic evaluation indicates that HSP110 forms a ring-like structure at the nucleolar periphery in the region of the nucleolar chromatin (Subjeck et

al., 1983; Kochevar et al., 1991). Depletion of this protein in yeast abolished the ability of these cells to undergo induced thermotolerance (Sanchez & Lindquist, 1990; Kochevar et al., 1991). Specific functions remain undefined but based upon its location following heat shock this protein may protect ribosomal assembly in the nucleolus.

HSP90

HSP90 is a heat-inducible protein that has a molecular weight ranging from 83 to 94 kDa and a pI of 5.1 to 5.8 (Welch et al., 1983; Moore et al., 1987; Iwasaki et al., 1989). A glucose-regulated protein (GRP94) is also a member of the HSP90 family of proteins; localization is restricted to the endoplasmic reticulum and Golgi apparatus (Kochevar et al., 1991). In solution, HSP90 is phosphorylated and exists as a dimer derived from distinct genes (Welch et al., 1983; Rebbe et al., 1987; Hickey et al., 1989). HSP90 associates with a variety of intracellular proteins including tyrosine kinases (Craig & Lindquist, 1988), oncogene products such as $pp60^{v\text{-}src}$, cytoskeletal elements such as actin and tubulin (Koyasu et al., 1986) and steroid receptors (Smith & Toft, 1993).

In particular, HSP90 has been immunolocalized and crosslinked with glucocorticoid, progesterone, estrogen and androgen receptors (Smith & Toft, 1993). The monomeric stoichiometry of HSP90 in receptor complexes is usually 2:1 (Bresnick et al., 1990; Smith & Toft, 1993). Binding of HSP90 is proposed to dictate a conformation of the steroid receptor that is competent to bind ligand. Several lines of evidence exist to support this thesis. Steroid hormone binding activity is directly correlated with high levels of HSP90 (Bresnick et al., 1990; Pratt et al., 1992). Moreover, functional glucocorticoid receptors are not present in yeast mutants having

low expression of HSP90 and glucocorticoid receptors devoid of HSP90 can not bind ligand (Picard et al., 1990; Smith & Toft, 1993). Finally, reconstitution of glucocorticoid receptor with HSP90 causes conversion of the receptor from a form with low affinity for steroid and high affinity for DNA response element to one that has a high affinity for steroid and incapable of binding DNA (Scherrer et al., 1990; Pratt et al., 1992). Following ligand binding, HSP90 dissociates from the receptor complex in an ATP-dependent manner since subsequent receptor complexes are deficient of bound HSP90 (Smith et al., 1992; Smith & Toft, 1993). Mechanisms associated with transcriptional competency of the receptor following ligand binding remain unclear, but appear to require more than dissociation of HSP90 (Bagchi et al., 1990; Baniahmad & Tsai, 1993).

A role of HSP90 during thermotolerance has been described and may in part due to an ability to associate with the cytoskeleton and inhibit translation. Treatment of mouse cells with antisense RNA, reduced growth of cells at 42°C (Bansal et al., 1991). Immunoprecipitation analysis indicates that HSP90 is complexed with cytoskeletal elements of the cell (Koyasu et al., 1986) and is capable of associating with eIF-2α (Rose et al., 1987).

HSP60

HSP60 is a heat-inducible mitochondrial protein that has a molecular weight ranging from 58 to 64 kDa with a pI of 5.8 (McMullin & Hallberg, 1988; Waldinger et al., 1989; Kochevar et al., 1991). HSP60 is immunologically and structurally related to the GroEL protein in *E. coli* and functions to fold mitochondrial proteins into their correct oligomeric structure (Craig et al., 1993; Hendrick & Hartl, 1995). Alterations in the gene sequence of HSP60 increased the incidence of malfolded proteins found within

the mitochondria (Cheng et al., 1989). Oligomeric assembly of mitochondrial proteins via association with HSP60 occurs in an ATP-dependent manner (reviewed by Craig et al., 1993; Hendrick & Hartl, 1995). Addition of ATP induced the release of mitochondrial proteins that were of the correct oligomeric structure; whereas, depletion of ATP increased the occurrence of newly imported proteins being complexed with HSP60 (Ostermann et al., 1989).

Actions of HSP60 are in part mediated by HSP70. In yeast, proteins following translocation are first associated with HSP70 and second with HSP60 (Craig et al., 1993). A role of HSP60 during thermotolerance has been described in *E. coli*, and may in part be due to an ability to maintain protein structure in the mitochondria following heat shock (Hendrick & Hartl, 1993). Martin et al. (1992) showed that HSP60 forms complexes during heat shock and prevents thermal deactivation of dihydrofolate.

HSP27

HSP27 is a family of proteins, encoded by separate genes, ranging in molecular weights of 25 to 30 kDa with a pI of 5.9 to 6.3 (Hickey et al., 1986; Arrigo & Welch, 1987; Faucher et al., 1993). With the exception of HSP26 (Lee et al., 1990), most of the proteins belonging to the HSP27 family are deficient or very low in methionine and are found in a phosphorylated form (Kochevar et al., 1991). HSP26 contains a substantial amount of methionine and is localized to the nucleus in a phosphorylated form (Lee et al., 1990). In contrast, HSP27 is found in the nonstressed cell as aggregates containing mRNA that are localized around the Golgi (Kochevar et al., 1991).

A role for HSP27 in thermotolerance has been described. Exposure of cells to heat shock results in heat-induced increases in HSP27 (Welch, 1985; Landry et al., 1989).

Also, several heat resistant variants of Chinese hamster ovary cells exhibited overexpression of HSP27 with no unusual modifications in the amounts of other heat shock proteins (Chretein & Landry, 1988). Similarly, overexpression of HSP27 via transfection, provided immediate protection to heat shock (Landry et al., 1989). Using four mammalian cell lines, Lee et al. (1990) noted that exposure of cells to heat shock reduced the levels of HSP26 found in nuclei by 30-70%. Restoration of this protein in the nucleus correlated with thermotolerance. Specific functions of this particular family of proteins during thermotolerance remain speculative.

Ubiquitin

Ubiquitin is a highly conserved 76 amino acid protein (~8.5 kDa) that is activated and bound by isopeptidic bonds to selected proteins which are to be degraded through nonlysosomal pathways (Kochevar et al., 1991; Craig et al., 1993). Within the cell, ubiquitin is found in a free form or associated with a variety of cellular proteins (Kochevar et al., 1991). Initial activation and binding of ubiquitin to lysine residues of selected proteins to be targeted for degradation is dependent upon a series of enzymatic reactions (Hershko, 1991; Craig et al., 1993). Binding of a single ubiquitin initiates poly-ubiquitination which in turn activates a multicatalytic high molecular weight protease, called a proteosome, that then degrades the selected protein to yield free amino acids and recyclable ubiquitin (Ciechanover et al., 1990; Craig et al., 1993). Proteins targeted for degradation via the ubiquitin pathway are characterized generally as having an amino-terminus deficient in methionine (Ciechanover et al., 1990) and containing basic residues such as histidine, arginine or lysine or hydrophobic residues such as leucine, tryptophan, phenylalanine or tyrosine (Reiss et al., 1988). Free intracellular ubiquitin decreases

following stress with a concomitant rise in ubiquitin conjugates in a process that presumably reflects accumulation of denatured proteins (Pratt et al., 1989; Kochevar et al., 1991).

Transcriptional Regulation of Heat Shock Proteins

In essentially all organisms, stress or heat-inducibility of heat shock proteins is regulated primarily at the level of transcription (Morimoto et al., 1992; Morimoto, 1993). Critical for transcriptional activation is the interaction of a heat shock factor (HSF) with a specific cis-acting DNA element termed heat shock element (HSE). Multiple copies of HSEs are located in the 5' flanking region of the promoter for all heat shock protein genes identified in prokaryotic and eukaryotic organisms (usually within the first 500 bp) and consist of a variable number of 5 bp units, nGAAn (Tanguay, 1988). These sequences may be arranged in a head to head (nGAAnnTTCn) or tail to tail (nTTCnnGAAn) fashion (Craig & Gross, 1991). Using 5' deletion mutations, Morimoto et al. (1989) demonstrated that only one intact HSE is required for heat-induced activation of the hsp70 gene. Similar findings were reported in *Drosophila* (Pelham, 1982). Multiple copies are required for maximal activation. For example, the transcriptional activation of the hsp70 gene following HSF binding of a single HSE was 10-fold lower than that of the wild-type gene which contains two HSEs (Kay et al., 1986; Tanguay, 1988). Moreover, transcriptional activation was greater than wild-type if constructions with four overlapping HSEs were made.

Localization and orientation of HSEs vary according to the gene and species of interest (Tanguay, 1988). In general, the relative position of HSEs is unimportant for promoter activity. However, increasing distances from the transcriptional start site may

decrease promoter strength (Amin et al., 1987). Footprinting studies demonstrated that the affinity of the proximal element of the hsp70 gene in *Drosophila* was higher for HSF than for more distal elements. Furthermore, the proximal HSE of *Drosophila's* small heat shock protein genes stimulated stronger expression than HSEs farther upstream (Pauli et al., 1986).

Differential responsiveness of proximal and distally located HSEs may be related to the presence of other cis-acting regulatory sequences located in the promoter region of heat shock protein genes. In addition to HSEs, the promoter region of the hsp70 gene contains two SP1 sites and CCAAT boxes and a serum response element (SRE; Wu et al., 1987; Tanguay, 1988; Morimoto et al., 1989). Possible effects of neighboring sequences are especially apparent for constitutively synthesized heat shock proteins. Heat shock cognate genes contain a variety of regulatory sequences in their promoter region and are generally considered to be minimally responsive to heat shock. Sorger and Pelham (1987) suggested that the accessibility of HSF for HSE might be modified by an SP1 site that overlaps the HSE. Further, deletion analysis of all upstream elements of the proximal HSE partially restored heat-inducibility, suggesting that HSEs in constitutively expressed heat shock protein genes are functional. Thus, the presence of other regulatory elements is important.

At normal temperatures, HSF is found throughout the cytoplasm and nucleus of the cell in a monomeric form and is generally considered to have a low binding affinity for HSEs (Morimoto, 1993). In response to heat shock, HSF oligomerizes, acquires the ability to bind to the HSE and is capable of activating transcription of all heat shock protein genes (Jakobsen & Pelham, 1988; 1991; Abravaya et al., 1991). This process is

dependent upon a number of inducible factors (Morimoto et al., 1992). In particular, data from yeast and *Drosophila* demonstrated that purified HSF from heat shocked cells is found as a trimer (Perisic et al., 1989; Sorger & Nelson, 1989). Moreover, overexpression of HSF in human cells resulted in a monomeric form which could then be induced to trimerize following heat shock (Rabindran et al., 1993). Similar findings were noted in *Xenopus* oocytes (Zhou et al., 1994). Following trimerization, HSF is transported to the nucleus where it can then bind to HSEs (Sarge et al., 1993). Binding of HSF to HSE however, does not always confer transcriptional activation. Using a gel mobility shift assay, Sorger et al. (1987) noted that control and heat shocked yeast cells yielded the same amount of HSE-binding activity; only mobility of HSF-HSE complexes on polyacrylamide gels was altered following heat shock. The differences in mobility could be reduced by the addition of a phosphatase, suggesting that there were additional steps in heat shock protein gene expression (Morimoto et al., 1992; Morimoto, 1993) such as phosphorylation. Indeed, further analysis revealed that HSF purified from yeast and human cells is phosphorylated in a stress dependent manner (Sorger et al., 1987).

Recent elucidation of the sequence and structure of HSF has greatly enhanced understanding of the requirements for the processes of trimerization, competency to bind HSEs and ultimately activate transcription of specific heat shock protein genes. Multiple forms with distinct functions have been identified in different species (Morimoto et al., 1992). For example, HSF is the product of a single gene in yeast and *Drosophila* (HSF1; Wiederrecht et al., 1988; Clos et al., 1990), two genes in humans (HSF1, HSF2; Rabindran et al., 1991) and mice (Sarge et al., 1991) and three genes in tomatoes (HSF1, HSF2, HSF3; Scharf et al., 1990) and chickens (Schuetz et al., 1991). Overall sequence

homology is low (~40%); nonetheless all share several conserved sequences. Specifically, sequence comparison demonstrated conservation in the amino-terminal region of HSF that includes the DNA-binding domain, three other regions that contain overlapping leucine zippers designated as LZ1, LZ2 and LZ3 and a 12-residue long region of homology in the carboxy-terminal region located downstream of LZ3 (Rabindran et al., 1991; 1993; Schuetz et al., 1991). Deletion and substitution analysis in any of the LZs caused constitutive oligomerization and DNA binding (Zhou et al., 1994) suggesting that these regions are important for maintaining the monomeric inactive form of hHSF. Similar findings were noted in yeast (Peteranderl & Nelson, 1992). The importance of trimerization remains to be determined but may enable the DNA-binding function of HSF by facilitating cooperative binding of monomeric DNA-binding domains to the HSE motif (Zhou et al., 1994). Specific sequences through which these molecules acquire transcriptional activation remain unknown.

Distinct functional differences for HSFs have been described. In general, HSF1 induces heat shock protein gene expression following heat shock while HSF2 is induced during hemin-induced differentiation of human K562 cells (Sistonen et al., 1992). Differential responses to heat shock or other stressors may be related to binding capabilities of the molecules. Using an in vitro translation system, Sarge et al. (1991) noted that mHSF1 is expressed as an inactive form that is activated to bind HSE following exposure to temperatures >41°C; mHSF2, in contrast, is expressed in a form that binds to DNA constitutively but loses it ability to bind at temperatures >41°C. Though apparently differentially activated, recent evidence indicates that HSF1 and HSF2 can synergistically induce hsp70 gene transcription (Sistonen et al., 1992). In

particular, simultaneous activation of both HSF2 and HSF1 in K562 cells subjected to hemin treatment followed by heat shock resulted in the synergistic induction of hsp70. Further studies are required to discern the level of complexity involved in the regulation of heat shock protein gene expression.

At the molecular level, cells respond very rapidly to heat shock. Binding of HSF to HSE, as determined by gel mobility shift assays, is detected within minutes (Perisic et al., 1989; Westwood et al., 1991; Morimoto, 1993; Sarge et al., 1993). This is followed by an increase in mRNA for heat shock protein genes (Abravaya et al., 1991). Such a rapid response is facilitated by the absence of intervening sequences (Wu et al., 1985; Hunt & Calderwood, 1990; Grosz et al., 1992). Abravaya et al. (1991) noted that attenuation of HSF activation and heat shock protein gene expression occurs only when mild heat shock temperatures (42°C) are given; increasing the temperature by 1°C elicited a higher level of activation that did not become attenuated during a 4 h heat shock.

Most of the conditions known to mediate stress-induced heat shock protein gene expression cause denaturation of preexisting proteins or of newly made intracellular proteins (Voellmy, 1984; Goff & Goldberg, 1985). Anatham et al. (1986) noted that the stress response could be elicited directly by co-injection of denatured proteins and heat shock protein genes into frog oocytes. This effect was not observed when proteins were co-injected in their native form. Further analysis demonstrated that protein denaturation per se is insufficient for expression of heat shock protein genes; instead, Mifflin and Cohen (1994a) showed that aggregation distinguishes the proteins that are effective

inducers. Formation of large aggregates of denatured proteins positively correlated with the response.

Factors involved in the attenuation of the stress response following heat shock remain to be determined but recent evidence suggests a role for members of the heat shock protein family, in particular, HSC70, via an autoregulatory feedback loop (Morimoto et al., 1992). Decreases in mRNA for HSP70 in *Drosophila* (Craig & Gross, 1991) and yeast (Amaral et al., 1993) are coincident with increased levels of HSP70. Amaral et al. (1993) showed that decreases in mRNA for HSP70 could be inhibited if cells were incubated in the presence of a protein synthesis inhibitor such as cycloheximide and suggested that a protein non-existent before stress is involved in negatively regulating hsp70 expression. More direct evidence that HSP70 acts as a negative regulator of heat shock protein gene expression was reported by Mosser et al. (1993). In particular, constitutive overexpression of HSP70 in transfected human cells reduced the extent of HSF activation following heat shock. This inhibitory effect of HSP70 may (Abravaya et al., 1992) or may not (Rabindran et al., 1994) be relieved by addition of ATP. Studies using coimmunoprecipitation procedures and gel shift assays have demonstrated that HSP70 may be complexed with the latent (Rabindran et al., 1994), heat-activated forms of HSF (Baler et al., 1992) as well as denatured aggregates of proteins (Mifflen & Cohen, 1994b). Interestingly, injections of HSC70 into *Xenopus* oocytes lowers the stress response to both a thermal shock and to co-injected protein inducers (Mifflen & Cohen, 1994b).

Collectively, the following model has been proposed by Morimoto et al. (1992), Morimoto (1993), Mifflen and Cohen (1994b) and Ezzell (1995) to explain

autoregulation of HSP70 gene expression. At normal temperatures, HSF exists within the cytoplasm in a monomeric form bound to HSP70 proteins. Exposure to heat shock or other stressors creates a pool of denatured or malfolded cytoplasmic proteins which compete with HSF for association with HSP70. Dissociation of HSP70 with HSF removes the inhibitory regulatory influence on HSE binding. Thereafter, HSF trimerizes, is translocated to the nucleus where it becomes phosphorylated and binds HSE located within the promoter region of HSP70. Activation of HSF binding leads to elevated transcription, synthesis and accumulation of HSP70. Ultimately, the denatured proteins are either degraded or folded properly such that more HSP70 is able to reassociate with HSF and inactivate it.

Although there are many similarities for regulation of heat shock protein gene regulation in higher eukaryotes and *Drosophila*, there is at least one distinguishing feature worth mentioning. In contrast to mammalian cells, regulation of HSP70 gene expression is controlled at the level of elongation, not at the point of initiation of transcription (Krumm et al., 1993). UV-crosslinking and nuclear run-on assays revealed that a molecule of RNA polymerase II is bound to DNA near the transcription start site (O'Brien & Lis, 1991). At normal temperatures this polymerase has initiated transcription but pauses approximately 25 bases upstream of the promoter (Rougvie & Lis, 1988). Similar finding were noted for HSP27 and HSP26 (Rasmussen & Lis, 1993). Exposure to heat shock releases the pausity of the polymerase and allows for elongation of the transcript. Pausity of RNA polymerase II may be regulated by specific cis-acting elements upstream of the transcription initiation site. The promoter region of hsp70 in *Drosophila* is characterized by the presence of a TATA box, one HSE and a GAGA box

(Karch et al., 1981; Southgate et al., 1983). Multiple mutations in the GAGA box markedly reduced pausing of RNA polymerase II. GAGA factor is constitutively expressed in *Drosophila* cells; its association with the GAGA box has been shown to prevent association of nucleosomes or histone H1 with DNA (Krumm et al., 1993). Thus, GAGA boxes may function to initiate the formation of an open chromatin structure and allow for access of RNA polymerase II to the promoter region of heat shock genes. Similar mechanisms for the control of transcriptional elongation have been observed in the human and murine c-myc gene (Krumm et al., 1993). This mechanism does not appear to be operational for heat shock protein genes in higher eukaryotic cells in that the GAGA box is not present in the promoter region of heat shock protein genes and no reports exist to indicate that RNA polymerase II pauses near the transcription start site.

Translational Regulation of Heat Shock Proteins

Within minutes following exposure to elevated temperatures > 43°C, there is a shift in the translational specificity of cellular mRNAs. In particular, translation of most preexisting mRNAs within the cell is shutoff; whereas, heat-inducible heat shock protein mRNAs are preferentially translated (Duncan & Hershey, 1989; Pardue et al., 1992; Nover & Scharf, 1991). Inhibition of protein synthesis is not due to degradation of preexisting mRNAs (reviewed by Sierra & Zapata, 1994). For example, cellular mRNA extracted from heat shocked cells can be translated in vitro (Scott & Pardue, 1981) and the overall rate of translation can be restored following return of cells to normal temperatures in the presence of a known RNA synthesis inhibitor, actinomycin-D (Duncan & Hershey, 1989). Theoretically, inhibitory effects of heat shock on protein synthesis could in part be due to changes in the maturation of rRNA (Pardue et al., 1992),

RNA splicing (Yost et al., 1990) or polysome formation (Duncan & Hershey, 1989), alterations in cytoskeletal organization (Welch & Suhan, 1986; Wiegant et al., 1987) and translational machinery (Duncan & Hershey, 1989; Sierra & Zapata, 1994).

Initiation of protein synthesis is dependent upon four successive phases (reviewed by Morley, 1994): 1) association of the eukaryotic initiation factor, eIF-2α, and GTP with the initiator methionyl tRNA, 2) binding of 40S ribosomal subunit, 3) binding of the mRNA to be translated and finally 4) binding of the 60S ribosomal subunit, followed by release of initiation factors to form a complex referred to as the 80S initiation complex. Also, translation of mRNA is dependent upon the association of initiation factors with the cap structure of the mRNA. These factors are collectively referred to as eIF-4F and are important because they are required for unwinding of the secondary structure upstream of the start codon AUG (Kozak, 1980; 1989; Morley, 1994).

Heat-induced inhibition of protein synthesis may in part be due to alterations in the phosphorylation patterns of specific eukaryotic translation initiation factors. For example, increased phosphorylation of the eukaryotic initiation factor eIF-2α occurred coincident with heat-induced inhibition of protein synthesis in HeLa cells was (Duncan & Hershey, 1989). Modifications were proportional to the severity of heat shock. The importance of phosphorylation in the control of protein synthesis was demonstrated by the finding that expression of a serine to alanine phosphorylation resistant mutant at residue 51 of eIF-2α partially ameliorated heat-induced inhibition of protein synthesis in CHO cells (Murtha-Riel et al., 1993). Stimulation of eIF-2α phosphorylation was inhibited by a monoclonal antibody specific for a heme regulated eIF-2α kinase (HRI; Matts et al., 1993). Factors involved in the activation of such a kinase remain undefined

but may involve increases in denatured proteins that occur as a result of exposure of cells to elevated temperatures. Denatured proteins increased the phosphorylation of eIF-2α and reduced overall protein synthesis (Matts et al., 1993). The concentration of denatured bovine serum albumin (BSA) required to inhibit protein synthesis correlated with levels of HSP70 present in lysate. In this experiment, HSP70 coimmunoabsorbed with denatured BSA or HRI from the lysates that were administered native BSA.

Inhibition of translation by heat shock is also accompanied by dephosphorylation of eukaryotic initiation factors involved in cap recognition of cellular mRNAs. In particular, exposure of HeLa cells to temperatures > 42°C resulted in an accumulation of nonphosphorylated eIF-4B and eIF-4F (Duncan & Hershey, 1989; Duncan et al., 1987). The functional significance of the effects of heat shock on eIF-4B remain unresolved but it is clear that severe heat shock has important effects on the complex of initiation factors involved in cap recognition. Addition of purified eIF-4F to heat shocked Ehrlich ascites tumor cells was sufficient to restore translation of normal cellular mRNAs (Panniers et al., 1985).

The mechanisms responsible for preferential translation of heat shock protein mRNAs remain undefined. Of principle importance are intrinsic structural properties that are unique to heat shock protein mRNAs. Heat-inducible heat shock protein mRNAs are not capped and have unusually long 5' untranslated leader sequences that confer preferential translation (Holmgren et al., 1981; DiNocera & Dawid, 1983; Klementz et al., 1985). Lindquist and Petersen (1990) demonstrated that this sequence is very rich in adenosines and G-C poor resulting in a structure that is relatively free of secondary structure and thus may favor preferential translation in heat shocked cells in which the

function of eIF-4F (a complex of initiation factors required for recognition of the cap structure of most mRNAs) has been compromised. This model parallels the mechanism for translation of polio virus (Jackson et al., 1991; Morley, 1994) where infection causes inactivation of the essential cap binding protein, eIF-4F. Uncapped polio virus escapes repression because translation of viral mRNA is conducted via processes independent of cap recognition.

Heat shock also regulates translation of HSP70 through mechanisms that are dependent upon the 3' untranslated region of the gene (Moseley et al., 1993). Following heat shock, COS-1 cells transfected with a CAT construct containing the HSP70 3' UTR showed increased CAT activity relative to β-globin. This effect paralleled increases in mRNA and heat-inducible HSP70 as detectable by Western blotting. Another possible effect of heat shock to increase the preferential translation of heat shock proteins mRNAs is to enhance overall stability of heat shock protein mRNAs. Dellavalle et al. (1994) demonstrated that the polyadenylation of hsp70 mRNAs varied with increasing temperature. Specifically, as temperature increased, a larger fraction of hsp70 mRNA was in a polyadenylated form; within 90 min following return of *Drosophila* cells to 25°C, the majority of the transcripts had short poly(A) tails or lacked poly(A) entirely. Exposure of cells to a severe heat shock markedly delayed deadenylation suggesting that adenylation/deadenylation may play a key role in regulating at least the synthesis of HSP70. In other systems, increasing length of the poly(A) tail is associated with increased stability of mRNA (Jackson & Standart, 1990; Bachvarova, 1992).

Developmental Regulation of HSP70 Synthesis

Constitutive and heat-induced gene expression and synthesis of members of the HSP70 family are regulated developmentally in a number of organisms. Synthesis of HSP70 has been characterized in mouse oocytes and preimplantation embryos and correlative evidence suggests a role for these proteins in the developmental acquisition of thermotolerance.

HSP70 Synthesis in Oocytes

Of the total pool of mRNA that is accumulated during oogenesis, large amounts of mRNA for cognate forms of HSP70 have been detected in *Drosophila* (Palter et al., 1986), *Xenopus* (Bienz, 1984) and murine (Zimmerman et al., 1983) oocytes. Using reverse transcription-polymerase chain reaction, Manejwala et al. (1991) demonstrated that levels of HSP70 in murine oocytes are high initially and then decrease rapidly following resumption of meiosis. Due to the highly conserved sequence homology of the cognate and heat-inducible hsp70 genes, Zimmerman and Cohil (1991) pointed out that it is unclear whether the hsp70 mRNA reported by Manejwala et al. (1991) is actually hsc70 mRNA.

During oogenesis in the mouse, developmental regulation of the synthesis of heat shock cognate (HSC70) and heat-inducible (HSP68) proteins has been described. Using comigration on 2-D SDS-PAGE and protease digestions, Curci et al. (1987) demonstrated that oocytes labeled with [^{35}S]methionine constitutively synthesize two heat shock proteins identified as HSP83 and HSC70. Moreover, heat-inducibility of heat shock proteins was dependent upon the developmental stage of the oocyte (Curci et al., 1991). For example, exposure of oocytes during the growth phase to 43°C for 20-30 min

resulted in the induction of two isoforms of a heat shock protein corresponding to 68 kDa (HSP68). Heat-induction of this protein was dependent upon new transcription since addition of 0.5-1 µg/mL actinomycin-D or 10 µg/mL α-amanitin blocked heat-induced increases. In contrast, preovulatory stage oocytes lacked the ability to synthesize heat-inducible forms of HSP68 (Curci et al., 1987). Cumulatively, data indicated that the heat shock response of mouse dictyate oocytes is dependent upon the differentiative status of the follicle and oocyte.

Xenopus oocytes also contain a significant quantity of hsp70 mRNA (Bienz, 1984). It has been reported that this message is only translated when oocytes are exposed to elevated temperature (Bienz & Gurdon, 1982). Authors speculated that the heat shock mRNA was stored in an inactive state by a "masking" mechanism. However, these results were not reproducible and since have been considered to be due to the contamination of follicular cells (King & Davis, 1987; Nover & Scharf, 1991). Analysis of developmental and heat-inducibility of other heat shock genes has been most extensively performed in *Drosophila*. In particular, subsets of the low molecular weight heat shock protein gene set (hsp83, hsp28 and hsp26), were shown to be transcribed in ovarian nurse cells, accumulate in developing oocytes (Zimmerman et al., 1983) and later be utilized by the early embryo (Palter et al., 1986).

HSP70 Synthesis in Preimplantation Mouse Embryos

hsp70 is one of the earliest transcripts produced following fertilization (Bensuade et al., 1983; Christians et al., 1995). Developmentally regulated increases in this transcript occurs in an α-amanitin sensitive manner (Bensaude et al., 1983; Manejwala et al., 1991; Christians et al., 1995). Expression of hsp70 has been characterized as a

landmark of early zygotic activity and is restricted to the first burst of transcription occurring between the late 1-cell stage and continuing to the 2-cell stage of development in mouse embryos. Thereafter, expression is repressed before the completion of the second round of DNA replication (Christians et al., 1995), presumably due to maturation of chromatin structure (Thompson et al., 1995).

The initiation of hsp70 synthesis during embryonic genome activation does not require heat shock; however, the degree of expression is influenced by environmental conditions. In particular, the increase in hsp70 expression was greater for cultured embryos than for those that developed in utero; addition of antioxidants reduced slightly the magnitude of expression seen in cultured embryos (Christians et al., 1995).

The ontogeny of the synthesis of members of the HSP70 family in murine embryos has been described using metabolic labeling with [^{35}S]methionine and 1-D and 2-D SDS-PAGE. Two cell embryos cultured at 37°C synthesize two cognate HSC70 molecules, HSC74 and HSC70 (Hahnel et al., 1986). At the 8-cell and blastocyst stage, constitutive synthesis of HSC70 continues. When embryos are exposed to 43°C, a third protein, designated as HSP68, is synthesized but only at the late morula or blastocyst stage of development (Wittig et al., 1983; Morange et al., 1984; Muller et al., 1985; Hahnel et al., 1986; Heikkila et al., 1986). Using the transgenic mouse model, Bevilacqua et al. (1995) demonstrated that developmental activity of a full length hsp68 construct was spontaneously activated at the 2-cell stage, attenuated at the 4-cell stage and heat-inducible at the 16 to 32-cell stage. Moreover, prior to differentiation, embryonic stem cells are incapable of synthesizing HSP70 in response to heat shock (Morange et al., 1984; Wittig et al., 1983). These studies suggest that synthesis of heat-

induced HSP70 is dependent upon cellular differentiation that occurs at the early morula and blastocyst stages of development.

Heat Shock Protein Synthesis in Postimplantation Embryos

Evidence from the rat suggests that postimplantation embryos are also capable of synthesizing a variety of heat shock proteins. Mirkes (1987) demonstrated that exposure of rat embryos on d 10 of pregnancy to culture temperatures ranging from 42 to 43°C induced synthesis of specific proteins with molecular weights ranging from 28-82 kDa. Exposure of embryos to 43°C increased synthesis of eight heat-inducible proteins while exposure to 42°C induced only 3 heat shock proteins. Of the inducible proteins, HSP70 was the predominant protein. Heat inducibility of these proteins appear to be developmentally regulated in the rat (Mirkes et al., 1991) because the heat shock response in rat embryos on d 9 is characterized by the synthesis of heat shock proteins corresponding to the M_r of 28-78 kDa; by d 10 two additional heat shock proteins were noted. In contrast, exposure of embryos to heat shock on d 11 only resulted in the synthesis of 3 heat shock proteins. No heat-inducible heat shock proteins were noted by d 12.

Expression of hsp70 in most cells is stimulated by the binding of the trimerized transcription factor HSF to the conserved regulatory heat shock elements located upstream of the TATA box (HSE; Morimoto, 1993). Mezger et al. (1994) designed an experiment to examine the profile of HSE-binding activities in mature oocytes and during preimplantation development of mouse embryos using gel shift assays. Exposure of 1-cell and 2-cell embryos to heat shock resulted in strong HSE-binding activity. At the 4-cell stage, no binding activity was noted. Thereafter, a progressive reappearance of the

ability to induce HSE-binding activity was observed between the 8-cell and blastocyst stage of development. Heat-induced binding activity at the blastocyst stage parallels the appearance of heat shock gene inducibility (Christians et al., 1995). When embryos were cultured at 37°C, HSE-binding activity was not detected until the morula stage. Results suggested that the absence of heat-inducibility of hsp70 gene expression in the 2-cell embryo is not limited by the absence of HSFs but may be related to progressive changes in chromatin structure as assessed using a luciferase transgene with or without flanking scaffold attachment regions in the early embryo (Thompson et al., 1995).

Using crosslinking and antisera specific for HSF1 and HSF2, Murphy et al. (1994) demonstrated that most of the constitutive HSE-binding activity binding reported by Mezger et al. (1994) is in large part due to HSF2; whereas heat-inducible binding is predominantly due to a trimerized HSF1. Interestingly, at normal temperatures HSF2 is found in an oligomeric form and is quantitatively higher in totipotent embryonal cells compared to nonembryonal cells lines (Murphy et al., 1994). Thus, HSF2 may have a role during mouse embryogenesis.

Role of HSP70 in Developmental Acquisition of Thermotolerance

There is generally a very close relationship between the ability of a cell to synthesize heat shock proteins and withstand elevations in temperature. For example, Curci et al. (1991) demonstrated that growing oocytes, which are capable of synthesizing increased amounts of heat-induced HSP70, progressed to metaphase II in greater numbers following heat shock than was the case for preovulatory oocytes which are transcriptionally incompetent. The oocyte's somatic counterparts synthesized increased amounts of the typical murine heat shock proteins, such as HSP110, HSP89, HSP70,

HSP68 isoforms and HSP33 (Curci et al., 1987). Furthermore, microinjection of HSP70 mRNA increased thermoresistance in mouse oocytes exposed to 43°C (Hendrey & Kola, 1991). Ability to withstand elevated temperatures in mouse embryos cannot be achieved until the blastocyst stage, at which time HSP70 can be transcriptionally induced (Hahnel et al., 1986; Christians et al., 1995). A similar relationship has been described in rat embryos (Walsh et al., 1987, 1989). Constitutively synthesized HSP70 molecules in early embryos are generally not increased in response to heat shock (Hahnel et al., 1986) and it is at these particular stages that embryos and oocytes are very sensitive to elevated temperatures.

Role of Glutathione and Production of Free Radicals During Early Embryonic Development

Early cleavage stage embryos are subject to the deleterious effects of free radicals and their survival is dependent upon an ability to utilize defense systems to eliminate these noxious molecules. Recent evidence suggests that the antioxidant, GSH, is important during preimplantation mammalian development and may ameliorate some of the inhibitory effects of free radicals that are produced during heat shock and as a result of increased oxygen tension during culture.

Glutathione

Glutathione is an antioxidant that functions to scavenge free radicals. Synthesis of this molecule occurs in the cytoplasm of the cell and involves two consecutive enzymatic reactions (Bray & Taylor, 1993). In the first reaction, an amide bond between glutamic acid and cysteine is catalyzed by γ-glutamylcysteine synthetase. Lastly, addition of glycine is catalyzed by glutathione synthetase. The resulting structure is a tripeptide with the sequence of γ-glu-cys-gly. The rate limiting enzymatic step in the

production of GSH is γ-glutamylcysteine synthetase; buthionine sulfoximine (BSO) is a selective inhibitor of this enzyme (Griffith & Meister, 1979a; 1979b).

The content of GSH within the cell usually exceeds mM concentrations (Allen, 1991). Greater than 90% of the intracellular stores of GSH is found in a reduced form (GSH; Griffith & Meister, 1979a). During oxidative stress, GSH limits deleterious effects of free radicals by acting as a substrate for enzymes such as glutathione peroxidase and catalase (Bray & Taylor, 1993) allowing for conversion of reactive free radicals such as H_2O_2 to H_2O. Consequently, GSH is oxidized to glutathione disulfide (GSSG) which may be exported from the cell or converted back to reduced GSH via glutathione reductase (Bray & Taylor, 1993).

Role of Glutathione and Free Radicals During Early Embryonic Development

Synthesis of GSH by the oocyte during maturation has been reported in the mouse (Calvin et al., 1986) pig (Yoshida et al., 1993) and hamster (Perreault et al., 1988) and is required for decondensation of sperm chromatin and pronuclear formation following fertilization (Perreault et al., 1984; Calvin et al., 1986; Perreault et al., 1988; Yoshida et al., 1993; Funahashi et al., 1994). Injections of BSO given subcutaneously in the abdominal region of mice 2 days prior to ovulation prevented fertilization and subsequent development of the few ova that were fertilized (Calvin et al., 1986). In contrast, addition of molecules to maturation medium that are known to increase GSH synthesis improved pronuclear formation in pig oocytes (Yoshida et al., 1993) and development to the blastocyst stage following fertilization in bovine (Matos et al., 1995) and murine oocytes (Gardiner & Reed, 1995).

Following resumption of meiosis, GSH content in the mouse decreases continuously to the blastocyst stage (7 mM to 0.7 mM; Gardiner & Reed, 1994). Ability to synthesize increased amounts of GSH is not noted until the blastocyst stage (Gardiner & Reed, 1995). Stores of GSH in the unfertilized oocyte are believed to exceed that required for fertilization and may be required for cleavage in the early embryo (Gardiner & Reed, 1994).

Synthesis of glutathione is also required during later stages of development. Depletion of this molecule at 10.5 d of pregnancy using BSO increased the number of dead and malformed embryos (Hales & Brown, 1991). Specific malformations included blebs of the maxillary or nasal processes, prosencephalon or forelimb buds and small or misshapen heads (Slott & Hales, 1987).

Although the deleterious effects of free radicals have been noted, it is important to recognize that these molecules also have a role during development. For example, H_2O_2 present in the blastoceolic fluid of the blastocyst is believed to cause apoptosis of pretrophectodermal cells whereas the cells that are destined to become the embryo may be protected from this molecule via mechanisms that utilize GSH (Pierce et al., 1991).

Thermoprotective Effects of Glutathione and its Role in Developmental Acquisition of Thermotolerance

Detrimental effects of heat shock on embryonic development are in part due to increased generation of free radicals and peroxides (Loven, 1988; Ealy et al., 1992; Aréchiga et al., 1994; 1995). Supplementation of GSH to culture medium increased resistance of bovine embryos to 42°C (Ealy et al., 1992). Similarly, administration of S-adenosyl-L-methionine, an inducer of glutathione synthesis, decreased the deleterious effects of 43°C for 2 h on viability of morula and development to blastocyst stage

(Aréchiga et al., 1995). In contrast, beneficial effects of GSH, taurine and glutathione ester on subsequent development was not noted when 2-cell bovine embryos were exposed to a mild heat shock of 41°C (Ealy et al., 1995). Perhaps disparity of the thermoprotective effects of GSH administration during heat shock is due to differences in the developmental stages examined or on the severity of heat shock. The converse is true when early embryos are depleted of glutathione during heat shock. Culture of embryos with BSO reduced the ability of murine morula to undergo induced thermotolerance (a phenomenon whereby exposure to a mild heat shock makes cells more resistance to a subsequent more severe heat shock) (Aréchiga et al., 1995).

Correlative evidence in the mouse embryo suggests a role of GSH in the developmental acquisition of thermotolerance. Ability to synthesize increased amounts of GSH is first noted at the blastocyst stage (Gardiner & Reed, 1995) which is coincident with the ability of the early embryo to withstand elevations in temperature (43°C; Muller et al., 1985). In contrast, early cleavage stage embryos are incapable of synthesizing GSH and are very sensitive to elevated temperatures (reviewed in Section I) and oxidative stress (Gardiner & Reed, 1994).

Effects of Free Radicals and Glutathione During Developmental Blocks

Oxygen tension averages 8% in the rabbit oviduct (Mastroanni & Jones, 1965) and rat uterus (Yochim & Mitchell, 1968). Increasing oxygen tension during culture dramatically reduces the percentage of oocytes that survive in culture, reduces the number that resume meiosis and then cleave to the 2-cell stage following fertilization and reduces the number of 2-cell embryos that develop to the blastocyst stage (Eppig & Wigglesworth, 1995).

Failure of cultured zygotes to develop beyond a specific stage of development is commonly referred to as a developmental block. Developmental blocks occur at the 8-16-cell stage in bovine embryos (Camous et al., 1984) and at the 2-cell stage in mice (Goddard & Pratt, 1983). Factors associated with developmental blocks have been best studied in the mouse. Whitten (1971) first reported successful culture of mouse embryos beyond the 2-cell stage using a hypoxic gas mixture consisting of 5% oxygen, 5% carbon dioxide and 90% nitrogen. Enhanced development as a consequence of lowering oxygen concentration during culture has been also been reported for hamsters (McKiernan & Bavister, 1990), sheep and cows (Tervit et al., 1972; Thompson et al., 1990) and goats (Batt et al., 1991). In mice however, deleterious effects of oxygen tension may be dependent upon the differences in mouse strains and culture medium because Cross and Brinster (1973) reported the successful culture of early embryos using a normally oxygenated atmosphere of 5% CO_2 in air.

Addition of free radical scavengers to culture medium ameliorated the 2-cell block in mouse embryos (Legge & Sellens, 1991). In this study, addition of 1 mM GSH improved the development of mouse zygotes through the 2-cell block to the morula or blastocyst stage. Similarly, addition of cysteamine to maturation or culture medium increased GSH concentrations and promoted development of bovine oocytes and 6 to 8-cell embryos to the blastocyst stage (Matos et al., 1993; Takahashi et al., 1993). In contrast, other studies have not shown beneficial effects of GSH on embryo development (Nasr-Esfahani et al., 1990; Ealy et al., 1995) indicating that developmental blocks may be the result of several different mechanisms.

Embryonic Genome Activation

In most species, early cleavage stage embryos are transcriptionally incompetent and must rely on maternal pools of mRNA stored during oogenesis for the synthesis of proteins. For continued survival, the embryo must acquire the ability to transcribe and translate specific mRNAs. Failure to do so inhibits continued development. The transition from control by maternally inherited molecules to that of embryonic genome-derived transcription products is referred to as embryonic genome activation (EGA). The period of time during development when embryos acquire the ability to express genes of embryonic origin differs from one species to another (reviewed by Telford et al., 1990). During oogenesis, the growing oocyte accumulates pools of mRNA (Bachvarova, 1985; Schultz, 1986). Following resumption of meiosis, synthesis of mRNA ceases and accumulated stores begin to decline in a precipitous manner. Synthesis of new mRNA at the onset of EGA is critical for continued development because embryos that fail to undergo EGA do not undergo further cleavage (Schultz et al., 1995). This review will concentrate on the timing of EGA of various species and potential mechanisms associated with the maternal to zygotic transition.

Timing of Embryonic Genome Activation

The most detailed information available on mechanisms regulating EGA is for the mouse model. In this species, EGA occurs in two successive phases beginning at the late 1-cell and 2-cell stage of development (Christians et al., 1995; Schultz et al., 1995). The first phase is characterized by the presence of functional RNA polymerase II activity and transcription factors and precedes a second phase which is characterized by a marked transition in polypeptide synthesis (Conover et al., 1991; Latham et al., 1992). Tightly

coupled to the second phase is the expression of a large complex of 70 kDa polypeptides referred to as transcriptional requiring complex (TRC; Flach et al., 1982; Conover et al., 1991) as well as HSP70 (Bensaude et al., 1983; Christians et al., 1995). Synthesis of these proteins are more prominent in the 2-cell embryo and are repressed before completion of the second round of DNA replication (Conover et al., 1991; Christians et al., 1995). Additionally, PCR analysis of cDNAs generated from early embryos during this time revealed the presence of cDNAs that are detected only at the 2-cell stage of development (Rothstein et al., 1992). One of these amplicons corresponds to the protein synthesis translation initiation factor eIF-4C (Schultz et al., 1995). Transcripts of eIF-4C increase between the 1-cell and 2-cell stages and then decrease thereafter. Treatment of embryos with α-amanitin inhibits synthesis of the aforementioned polypeptides and further development beyond the 2-cell stage in the mouse (Bolton et al., 1984; Conover et al., 1991).

Much less is known about the period during development when bovine embryos make the transition from the maternal to zygotic control of gene activation. Initial studies indicated that EGA occurs at the 8 to 16-cell stage (reviewed by Telford et al., 1990). Both suboptimal culture conditions (Camous et al., 1984; Jones & First, 1990) and treatment with α-amanitin blocks subsequent development at the 8 to 16-cell stage (Barnes & First, 1991). Coincident with this stage are marked changes in [^{3}H]uridine incorporation (Camous et al., 1986), protein synthetic patterns (Frei et al., 1989), formation of functional nucleoli (King et al., 1988) and amounts of TCA-precipitable radioactivity (Frei et al., 1989). Even so, more recent studies utilizing embryos produced by in vitro fertilization suggests that EGA may occur earlier in development. Changes in

gene expression as assessed by [^{3}H]uridine incorporation and 2-D SDS-PAGE analysis of radiolabeled proteins have been reported as early as the 2 to 4-cell stage. More specifically, treatment of 2-cell embryos with α-amanitin blocked [^{3}H]uridine incorporation (Plante et al., 1994) and blocked the synthesis of 12 proteins at the 2-cell stage (Marcucio et al., 1995), 8 proteins at the late 4-cell stage and 23 proteins at the 8-cell stage (Barnes & First, 1991).

Disparity of results are difficult to explain and indicate that culture conditions may modulate gene expression. Vernet et al. (1993) reported that the transcriptional activity of early mouse embryos was affected by their manipulation and culture. Moreover, expression of HSP70.1 was much higher in embryos that developed in culture when compared to those developed in vivo (Christians et al., 1995). Similar methods as those mentioned previously have been employed to determine the timing of EGA in other species. The transition from the maternal to zygotic control is thought to occur at the 4-cell stage in pigs (Davis, 1985) and humans (Braude et al., 1988) and 8 to 16-cell stage in sheep (Crosby et al., 1988) and rabbits (Manes, 1973).

Mechanisms Associated with Embryonic Genome Activation

The exact mechanisms involved in the initiation of gene expression in the early embryo are not clearly understood. Recent evidence suggests that mechanisms regulating the onset of EGA may be dependent upon the timing during development when this transition takes place and thus may differ according to species of interest. Schultz et al. (1995) proposed that if a species inherits a functional transcriptional complex at the 1-cell stage, like that which occurs in the mouse embryo, then mechanisms associated with EGA may be dependent upon changes in chromatin structure that are coupled to DNA

replication. In contrast, if EGA occurs several cell cycles later, such as in the bovine, then changes in the activity of the transcriptional machinery may account for the onset of EGA. The following review details evidence for potential mechanisms responsible for EGA in the mouse and other domesticated species.

In the mouse, alterations in chromatin structure associated with DNA replication play a major role in determining onset of EGA (Schultz et al., 1995; Thompson et al., 1995). Several lines of evidence exist to support this thesis. Addition of a DNA synthesis inhibitor (aphidicolin) to 1-cell embryos prior to entry into the S phase substantially reduced the increase in eIF-4C and TRC ordinarily occurring in the 2-cell embryo; whereas addition during S phase had little effect (Schultz et al., 1995). Moreover, Thompson et al. (1995), using a luciferase transgene specific for HSP70.1 with or without flanking scaffold attachment regions, demonstrated that progressive maturation of chromatin structure was responsible for constitutive expression of HSP70.1 gene expression previously reported by Christians et al. (1995).

Degree of histone acetylation may also be involved in EGA. As mouse embryos proceed from the 1-cell to the 2-cell stage of development, histones become hyperacetylated (Schultz et al., 1995) which is coincident with the first burst of transcriptional activity. Other studies suggest the involvement of cytoplasmic factors in regulating EGA. Transfer and fusion of transcriptionally active blastomeres obtained from bovine morulae and blastocysts to enucleated oocytes abolished expression of TEC-3 (Van Stekelenburg-Hamers et al., 1994). Reexpression was not noted until nuclear transfer embryos developed to the morula stage, suggesting that expression of embryonic genes in the bovine may be dependent upon the dilution or depletion of inhibitory components within the cytoplasm following several cleavage divisions. EGA may also

be the result of events that evoke changes in the phosphorylation status of cytoplasmic proteins. Culture of 1-cell mouse embryos in the presence of H8 (an inhibitor of PKA) inhibited expression of TRC (Schwartz & Schultz, 1992).

Finally, a role of the cell-cycle in EGA in bovine embryos was suggested by Jones (1994). He hypothesized that cell cycle mediated events may directly regulate the initiation of chain elongation by the transcriptional complex through phosphorylation of the large subunit of RNA polymerase II. In bovine embryos at early cleavage stages, the second cell cycle lasts approximately 12 h and has no detectable G1 or G2 phase. In contrast, the third cycle lasts 14 h and has no G1 but a shortened G2 phase which may or may not be coincident with the first burst of transcription (Barnes and Eyestone, 1990). Jones (1994) demonstrated that bovine embryos synthesize a cell cycle control protein, cdc25, at the 1-, 2-, 4- and 8-cell stage. Synthesis of this protein was only noted at the 8-cell stage and suggested that synthesis by earlier stage embryos is a result of the translation of maternal transcripts inherited from the oocyte. To determine if the initiation of transcription in bovine embryos is subject to cell cycle control, Jones (1994) microinjected the gene for cdc25 into 1-cell embryos and assayed for an 8-cell specific message via nested RT-PCR. Microinjection of cdc25 delayed transcription of the 8-cell transcript and hastened cleavage. He also determined that RNA polymerase II exists as a nonphosphorylated inactive form in the transcriptionally inactive 2-cell embryo and as a phosphorylated active form in the transcriptionally active 8-cell embryo. Based upon these results, Jones (1994) speculated that high levels of cell cycle components inherited from the oocyte result in accelerated cell cycles that are indicative of a pattern of cellular phosphorylation in which RNA polymerase II is maintained in a nonphosphorylated inactive form. As maternal stores of the cell cycle components become depleted or

diluted, the cell cycles slow and the repression of the C-terminal domain kinases is relieved such that RNA polymerase II becomes activated via phosphorylation.

Summary: A Tentative Thesis for Explaining Developmental Acquisition of Thermotolerance

For this thesis it was hypothesized that an embryo's intolerance to elevated temperature during the early cleavage stages is related to an inability to synthesize thermoprotective molecules such as HSP70 (illustrated in Figure 2-1). Several lines of evidence exist to support this model (reviewed in Chapter 2). First, increased susceptibility of bovine embryos to elevated temperature prior to the 8 to 16-cell stage is coincident with the period during development when embryos are transcriptionally incompetent. Secondly, ability of bovine embryos to withstand elevations in temperature follows full activation of the embryonic genome, suggesting the acquirement of transcriptional dependent thermoprotective mechanisms. Third, thermotolerance in other cell types is closely associated with transcriptional dependent increases in HSP70 synthesis following exposure to elevated temperature. Finally, correlative evidence in the mouse suggests a role for HSP70 in developmental acquisition of thermotolerance. Heat-induced increases in HSP70 synthesis is only noted at the late morula or blastocyst stage of development which is coincident with the period of time during development when mouse embryos acquire the ability to withstand elevations in temperature. In contrast, embryos at earlier stages of development are very susceptible to deleterious effects of elevated temperature and incapable of synthesizing increased amounts of HSP70. Thus, the overall objectives of this dissertation were to further characterize the effects of heat shock on the oocyte and bovine embryo, with the specific intention of identifying whether developmental changes in resistance of embryos to heat shock are related to an ability to undergo heat-induced synthesis of the thermoprotective molecule HSP70.

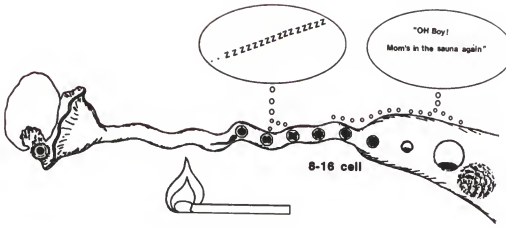

Figure 2-1. Model illustrating hypothesis of this thesis. Prior to the 8- to 16-cell stage, bovine embryos are transcriptionally incompetent (represented by ..zzzzzz..) and very susceptible to the effects of elevated temperature (lit match). Associated with full activation of the embryonic genome, later stage bovine embryos become more thermotolerant because of an ability to synthesize thermoprotective molecules such as HSP70 (represented by "OH Boy! Mom's in the sauna again).

CHAPTER 3
REGULATION OF HEAT SHOCK PROTEIN 70 SYNTHESIS BY HEAT SHOCK IN THE PREIMPLANTATION MURINE EMBRYO

Introduction

In many domestic animals, exposure to heat stress during early pregnancy causes embryonic mortality. The effects of heat stress decline as pregnancy progresses so that effects are minimal by d 3-5 in the ewe (Dutt, 1963) and cow (Ealy et al., 1993) and by d 5 in the pig (Tompkins et al., 1967). One possible explanation for this phenomenon is that embryos become more resistant to the deleterious effects of elevated temperatures as they advance in development. There is evidence for this in the cow (Ealy et al., 1993). The biochemical processes by which embryos develop resistance to elevated temperatures are not known; their identification could result in novel methods for protecting embryos from elevated temperatures. Tolerance to transient periods of elevated temperatures in many cells has been correlated with synthesis of a small subset of intracellular proteins known as HSPs (Riabowol et al., 1988; Hendrey & Kola, 1991; Nover & Scharf, 1991). Proteins of the HSP70 family include a constitutively expressed form HSC70 (Geibel et al., 1988) and a heat-inducible form HSP68 (Hunt & Calderwood, 1990). These proteins are produced in increased amounts in response to heat shock and have been demonstrated to play a protective role within the cell presumably through their ability to refold damaged proteins and protect RNA (Duncan & Hershey, 1989; Nover & Scharf, 1991). Neutralization of HSP70 with antibodies increased thermal sensitivity of fibroblasts (Riabowol et al., 1988) while microinjection

of HSP70 mRNA conferred thermal resistance in murine oocytes exposed to 42-43°C (Hendrey & Kola, 1991).

We have used the mouse as a model to study how embryos acquire thermotolerance during development. Preimplantation murine embryos first undergo induced thermotolerance (a phenomenon whereby prior exposure to a mild heat shock makes cells more resistant to a subsequent, more severe heat shock) at the 8-cell stage if developed in vitro, but not until the blastocyst stage of development if the embyros were developed in vivo. Induction of HSP70 synthesis by heat shock can occur by the late morula or blastocyst stage of development (Wittig et al., 1983; Morange et al., 1984; Muller et al., 1985; Hahnel et al., 1986; Heikkila et al., 1986), but it is not known whether the acquisition of induced thermotolerance in the embryo is caused by developmental changes in HSP70 synthesis in response to heat. The objectives of this study were to determine whether developmental patterns of heat-induced HSP70 synthesis are coincident with ontogeny of induced thermotolerance. Results indicate that heat-induced HSP70 synthesis occurs earlier in development than previously reported (Wittig et al., 1983; Morange et al., 1984; Muller et al., 1985; Hahnel et al., 1986; Heikkila et al., 1986), that the heat shock threshold for induction of HSP70 synthesis increases during development and that HSP70 synthesis occurs under conditions in which induced thermotolerance does not occur. These results indicate that other developmental changes are required for induced thermotolerance.

Materials and Methods

Embryo Collection and Culture

Mice (ICR strain; Harlan Sprague Dawley Inc., Indianapolis, IN, USA) were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Procedures for collection and culture of embryos were carried out as previously described (Ealy & Hansen, 1994). Briefly, embryos developed in vitro were collected at the 2 to 4 cell stage and cultured to the desired stage of development at 37°C. Cultures were performed in 5-7 µl microdrops of M16 medium containing 0.4% (w/v) bovine serum albumin that were covered with mineral oil. Embryos developed in vivo were obtained by sacrificing females and collecting embryos at appropriate times following injection of hCG (i.e., 8-cell embryos were obtained 63 h post-hCG and blastocysts at 96-100 h post-hCG). To reduce experimental variation, embryos that were developed in vitro and in vivo were synchronized for a given replicate such that both groups were at the same stage of development on the same day to which treatment was applied.

Heat Shock and Radiolabeling of Embryos

Embryos were randomly assigned within replicate to treatment and transferred to 50 µl of M16 medium ± 10% (v/v) htFCS and containing 50 µCi of a 70:30 [^{35}S]methionine and cysteine mixture (Amersham, Arlington Heights, IL; spec. act. > 1000 Ci/mmol; 12-24 8-cell embryos/microdrop; 6-21 blastocysts/microdrop). Control embryos were radiolabeled at 37°C for a total of 5 h and 20 min; heat-shocked embryos were radiolabeled at 40 or 43°C for 80 min and then at 37°C for 4 h. After labeling, embryos were washed three times in M16 medium containing 0.1% (w/v) polyvinyl alcohol and transferred in the smallest volume possible to 50 µl of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40, and 0.5% (w/v) dithiothreitol and frozen at -70°C until analysis.

Analysis of Radiolabeled Proteins

Incorporation of radiolabel into intracellular proteins was determined by TCA-precipitation (Mans & Novelli, 1961). Proteins from solubilized embryos were analyzed using 2-D SDS-PAGE, with isoelectric focusing in the first dimension [1% (v/v) each of two preblended ampholines (pH 3.5-9.5 and pH 5.0-8.0) from Pharmacia, Uppsala, Sweden] and SDS-PAGE [10% (v/v) polyacrylamide] in the second dimension (Roberts et al., 1984). Radiolabeled proteins were detected by fluorography. Each gel was loaded with 25-50,000 dpm of TCA-precipitable protein and exposed to x-ray film (Fuji; Tokyo, Japan); within each replicate, equal amounts of radioactivity were loaded and films were exposed for the same amount of time.

Statistical Analysis

TCA-precipitable radioactivity for control and heat-shocked embryos was expressed on a per embryo basis and analyzed by least squares analysis of variance using the General Linear Models procedure of SAS (1989). When heterogeneity of variance was present, actual means $\pm$ SEM were reported. Data were ranked and then ranks were analyzed by least squares analysis of variance.

Results

Exposure of embryos to a heat shock of 40°C did not affect overall ability to incorporate radiolabel into proteins during the course of treatment; a reduction in TCA-precipitable proteins was noted only when embryos were exposed to a heat shock of 43°C (Table 3-1). A representative fluorograph of proteins synthesized de novo for embryos radiolabeled at 37°C or while heat shocked is presented in Figure 3-1. Three different proteins corresponding to HSP70 were noted. Two proteins, designated HSC70

Table 3-1. Effect of heat shock on [^{35}S] labeled intracellular TCA-precipitable protein

Stage of development	Temper-ature	Number of Relicates	TCA-precipitable protein (dpm/embryo)	P-value
8-cell	37°C	4	6141 ± 2454[a]	NS
	40°C	4	4737 ± 2454	
Expanded blastocyst	37°C	3	67255 ± 11325	NS
	40°C	3	66354 ± 11325	
	37°C	3	40142 ± 19353[b]	P < 0.0001
	43°C	3	4373 ± 2146	

[a]Least squares means ± SEM
[b]Means ± SEM

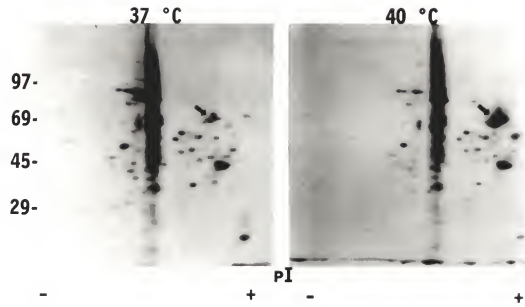

Figure 3-1. Representative profiles of [^{35}S]labeled proteins synthesized by 8-cell murine embryos that developed in vivo and were cultured at 37 or 40°C. De novo synthesis was assessed using 2-D SDS-PAGE and fluorography. For each gel, 25,000 dpm of TCA-precipitable radioactivity was loaded. Location of HSP70 molecules is indicated by arrows.

(M_r=69,906 ± 1016) and a slightly more basic HSC72 (M_r=72,432 ± 1271) were present in all embryos. The third protein, called HSP68, was of lower M_r (67,733 ± 1199) and more basic than HSC70 or HSC72 (Figure 3-2). HSP68 was generally absent in embryos cultured at 37°C (Figure 3-2) but was present in embryos exposed to heat shock.

Eight-cell embryos were examined for de novo synthesis of proteins as affected by temperature and type of development (in vitro versus in vivo). HSC70 and HSC72 were noted in all 8-cell embryos radiolabeled at 37 or 40°C; HSP68 was not present at 37°C with the exception that slight amounts were present in embryos developed in vivo (Figure 3-2). Exposure of embryos to 40°C generally increased synthesis of HSC70 and HSC72 as well as induced synthesis of HSP68 (Table 3-2). No differences in responses to heat shock were noted for embryos developed in vitro versus those that developed in vivo (Table 3-2; Figure 3-2).

Like the 8-cell embryo, HSC72 and HSC70 were present in expanded blastocysts at 37°C. HSP68 was not present at 37°C if embryos had developed in vitro; however, HSP68 was noted in expanded blastocysts cultured at 37°C and developed in vivo (data not shown). In contrast to the 8-cell embryo, exposure of expanded blastocysts to 40°C caused only slight or no change in synthesis of HSP70 molecules (Table 3-2; Figure 3-2). This was true for embryos that developed in vitro and in vivo and for embryos cultured with or without htFCS (Table 3-2). However, exposure to 43°C caused appearance of large amounts of radiolabeled HSP70 on fluorographs to produce a pattern similar to 8-cell embryos at 40°C (Table 3-2). In particular, 43°C caused appearance of HSP68; amounts of HSC70 and HSC72 were also enhanced as compared to 37°C (Figure 3-2; Table 3-2).

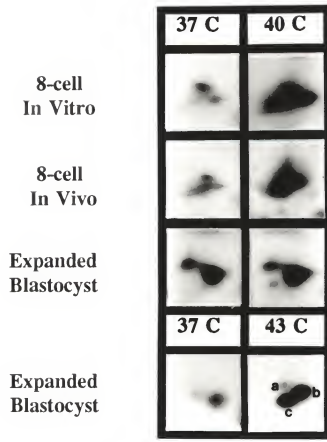

Figure 3-2. Representative profiles of [^{35}S]labeled HSP70 proteins synthesized by murine 8-cell embryos and expanded blastocysts cultured at 37, 40 (row 1-3) or 43 °C (row 4); proteins assessed using 2-D SDS-PAGE and fluorography. Each treatment (37 or 40 °C) applied to 8-cell embryos was replicated 3 times (in vitro developed) or performed once (in vivo developed); treatments (37, 40 or 43 °C) applied to expanded blastocysts were replicated 3 times. Within a replicate, equal amounts of TCA precipitable radioactivity (25-50,000 dpm) were loaded per gel. Shown is the region of the gel corresponding to HSC72 (a), HSC70 (b) and HSP68 (c).

Table 3-2. Effect of heat shock on synthesis of heat shock protein 70: summary of results from individual replicates[a]

Stage	Type of development	htFCS	# embryos (37°C / heat shock)	Heat shock (°C)	Intensity HSC70 37	HSC70 HS	HSC72 37	HSC72 HS	HSP68 37	HSP68 HS
8-cell	In vitro	-	19/16	40	+	---	-	---	-	---
8-cell	In vitro	-	20/24	40	+	--	-	--	-	--
8-cell	In vitro	-	24/23	40	--	--	-	--	-	-
8-cell	In vivo	+	12/15	40	+	----	-	----	-	----
EB[b]	In vitro	-	12/14	40	--	---	-	-	-	-
EB	In vitro	-	18/20	40	+	-	-	-	-	-
EB	In vivo	+	8/11	40	---	---	-	---	---	---
EB	In vitro	-	6/6	43	+	---	-	--	-	---
EB	In vitro	-	22/21	43	--	---	-	+	-	+
EB	In vitro	-	10/15	43	--	---	--	--	-	----

[a]in vivo developed embryos were were synchronized with one replicate of embryos developed in vitro such that both groups were at the same stage of development on the same day to which treatment was applied

[b]Expanded Blastocyst

-Protein absent

---- Very intense

--- Intense

-- Moderate intensity

+ Faint spot

Discussion

The M_r, isoelectric point and thermal induction of the proteins characterized in the present study are similar to murine HSP70 molecules previously reported (Nover & Scharf, 1991; Burel et al., 1992). The present finding that the 8-cell embryo can produce increased amounts of HSC72, HSC70 and HSP68 in response to a heat shock of 40°C is in contrast with previous reports that embryos are incapable of synthesizing HSP70 in response to a heat shock of 43°C until the late morula or blastocyst stage of development (Wittig et al., 1983; Morange et al., 1984; Muller et al., 1985; Hahnel et al., 1986; Heikkila et al., 1986). These earlier studies utilized heat shocks $\geq$ 43°C and this may have impaired the ability of cleavage-stage embryos to mount a heat shock response. Accordingly, heat shock of less severity, such as 40°C, may be required to adequately characterize the ontogeny of induced HSP70 synthesis in the murine embryo. Other evidence also exists that 8-cell embryos can undergo biochemical changes in response to 40°C because 8-cell embryos developed in vitro can undergo induced thermotolerance (Ealy & Hansen, 1994).

Within each replicate, a similar amount of TCA-precipitable radioactivity was analyzed by 2-D SDS-PAGE for control (37°C) and heat-shocked embryos. This was done to allow equivalent visualization of proteins at all temperatures. It is clear that changes in patterns of HSP70 in 8-cell embryos caused by exposure to 40°C represent alterations in synthesis rather than in the number of embryos loaded per gel because overall rates of protein synthesis were similar at 37 and 40°C. However, exposure of blastocysts to 43°C resulted in an 89% reduction in total TCA-precipitable protein. Accordingly, more embryos were used to prepare an electrophoretogram at 43°C than at 37°C. Thus, it is possible that the increase in the relative amounts of HSP70 molecules at 43°C may represent either 1) increased synthesis of the proteins by heat shock, as

reported in several cellular systems (Geibel et al., 1988; Nover & Scharf, 1991; Ealy & Hansen, 1994) or 2) a less-severe reduction in synthesis of HSP70 molecules in comparison with other intracellular proteins.

Molecules of the HSP70 family play a thermoprotective role since neutralization with antibodies increases thermal sensitivity of fibroblasts (Riabowol et al., 1988) and microinjection of HSP70 mRNA confers thermal resistance in murine oocytes exposed to 42-43°C (Hendrey & Kola, 1991). Nonetheless, present results indicate that induced thermotolerance in embryos requires more than HSP70 synthesis. Ealy and Hansen (1994) demonstrated that induction of thermotolerance in murine embryos was first noted at the 8-cell stage of development for embryos developed in vitro but not until the blastocyst stage for embryos developed in vivo. In the present study, 8-cell embryos synthesized increased amounts of HSC72, HSC70 and HSP68 in response to heat shock regardless of type of development. It is possible that there are subtle differences in the amounts of individual HSP70 molecules that could explain effects of type of development on induced thermotolerance responses that were not detectable using the present experimental approach. It is more likely, however, that these effects on embryonic capacity to undergo induced thermotolerance reflect changes in other biochemical systems. In this regards, DL-buthionine-[S,R]-sulfoximine (BSO) an inhibitor of glutathione synthesis, prevented induced thermotolerance in murine morula (Aréchiga et al., 1995). Moreover, Harris et al. (1991) demonstrated that BSO attenuated the thermotolerance response in postimplantation rat embryos without decreasing HSP70 mRNA or HSP70 synthesis.

While exposure of 8-cell embryos to 40°C increased HSP70 synthesis, this temperature was ineffective for expanded blastocysts; 43°C was required to cause large appearances of HSP70 on the electrophoretogram. The signal for heat-shock induced

HSP70 synthesis involves increased accumulation of denatured proteins (Ananthan et al., 1986). Thus, present results indicate that embryonic proteins may be more thermosensitive to denaturation at the 8-cell stage than at the blastocyst stage of development. This developmental difference in protein stability to elevated temperature, possibly caused by association of intracellular proteins with heat shock proteins or protection via antioxidants, could be one of the causes for the increase in resistance to elevated temperature exhibited as embryos progress through development (Ealy et al., 1993; Ealy & Hansen, 1994; Muller et al., 1985). The biochemical basis for this increased thermostability may also determine in part the developmental timing of induced thermotolerance responses.

CHAPTER 4
ELEVATED TEMPERATURE INCREASES HEAT SHOCK PROTEIN 70 SYNTHESIS IN BOVINE TWO-CELL EMBRYOS AND COMPROMISES FUNCTION OF MATURING OOCYTES

Introduction

Exposure of females to elevated temperatures is associated with increased early embryonic mortality. In most mammalian species, including cattle (Putney et al., 1989a; Ealy et al., 1993), pigs (Tompkins et al., 1967) and sheep (Dutt, 1963), deleterious effects of heat stress on embryonic mortality are more pronounced when occurring at or near the time of estrus than when occurring later. This result suggests that the maturing oocyte is very susceptible to heat stress. Moreover, exposure of maturing oocytes to elevated temperatures disrupted spindle formation during metaphase I in mice (Baumgartner & Chrisman, 1987) and reduced the frequency of oocytes that progressed to metaphase II in mice and cattle (Lenz et al., 1983; Baumgartner & Chrisman, 1987).

As embryos proceed in development, their ability to withstand elevations in culture temperature increases (Muller et al., 1985; Ealy et al., 1995). Acquisition of thermotolerance may be related to the ability to synthesize heat shock proteins in response to heat. In the mouse, preimplantation embryos can synthesize increased amounts of members of the HSP70 family in response to heat shock at least as early as the 8-cell stage (Chapter 4; Hahnel et al., 1986). Effects of heat shock have been noted for both the constitutively synthesized HSP70 variants HSC70 as well as the highly-inducible forms (designated as HSP68 in the mouse in Chapter 1). However, it is not

known how early in development heat-induced synthesis of HSP70 molecules occur. A thermoprotective role of HSP70 is well documented; immunoneutralization of HSP70 increased thermal sensitivity of fibroblasts (Riabowol et al., 1988) while microinjection of HSP70 mRNA conferred thermal resistance in murine oocytes exposed to 42-43°C (Hendrey & Kola, 1991). Molecules of the HSP70 family are believed to provide thermoprotection by refolding damaged proteins and protecting ribosomal RNA (Duncan & Hershey, 1989; Nover & Scharf, 1991).

The oocyte is intimately associated with its companion cumulus cells. This association is required for continued function of both the oocyte and cumulus cells (Buccione et al., 1990). However, specific signals and chemical contributions of the cumulus to the oocyte in the face of a changing environment remain to be determined. Perhaps cumulus cells aid the oocyte by protecting it from changes in its environment such as heat shock.

The objectives of the present study were threefold: 1) to evaluate effects of heat shock on oocyte function as assessed by cleavage and development and associated effects on membrane integrity and protein synthesis, 2) to determine whether responses of bovine oocytes to heat shock are modified by the presence of cumulus cells and 3) to determine if bovine oocytes and 2-cell embryos are capable of synthesizing heat shock proteins in response to heat shock.

Materials and Methods

Materials

Estradiol, fluorescein diacetate (FDA), heparin, HEPES, mineral oil, polyvinylalcohol, sodium pyruvate, antibiotics and TCM-199 were purchased from

Sigma Chemical Company (St. Louis, MO). Bovine steer serum and htFCS were purchased from Pel-Freez (Rogers, AK) and Atlanta Biologicals (Norcross, GA), respectively. Frozen semen from various bulls was obtained from American Breeders Service (Madison, WI). The CZB medium was prepared as described by Chatot et al. (1989), modified Tyrode's solutions (HEPES-TALP and IVF-TALP) were prepared as described by Parrish et al. (1986), and CR1aa medium as described by Rosenkrans et al. (1993). Sterile saline [0.9% (wt/vol) NaCl] was supplemented with 550 μg/L amphotericin, 100,000 IU/L penicillin-G and 100 mg/L streptomycin (ABAM) or with 100,000 IU/L penicillin-G and 100 mg/L streptomycin (Pen/Strep; Sigma Chemical). Pituitary-derived follicle stimulating hormone (FSH-P) was purchased from Schering (Kenilworth, NJ). Materials purchased from Fisher Scientific (Fair Lawn, NJ) included acrylamide solution (40%), ethidium bromide (EtBr), sodium salicylate and trichloroacetic acid (TCA). X-Ray film was from Fuji (Tokyo, Japan). Radiolabeled L-[^{35}S]methionine and L-[^{35}S]cysteine (70% L-[^{35}S]methionine and 15% L-[^{35}S]cysteine; specific activity > 1000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). Ampholines were purchased from Pharmacia (Uppsala, Sweden).

Collection of Oocytes

Ovaries were obtained at a local abattoir, transported to the laboratory in sterile saline [0.9% (w/v) NaCl] containing ABAM at 25-30°C and were then washed several times in sterile saline containing Pen/Strep. Cumulus oocyte complexes (COCs) were collected by making checkerboard incisions approximately 2 mm in width and depth on the ovarian surface and then washing the ovaries vigorously in collection medium [TCM-199 with Hank's salts, 10 mM HEPES, 2% (v/v) steer serum, 40 U/L heparin and 1%

ABAM]. Only oocytes with a dark, evenly granulated ooplasm and tightly compacted cumulus were used. Unless otherwise stated, COCs were used immediately following collection; COCs were placed in groups of 10 in 50 µl oocyte maturation medium [OMM; TCM-199 with Earle's salts, 10% (v/v) steer or fetal calf serum, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 2 µg/mL estradiol and 20 µg/mL FSH-P], covered with mineral oil and allowed to incubate for 24 h at assigned temperatures (39, 41 or 42°C) in an atmosphere of 5% CO_2.

Cleavage Rate and Subsequent Development to Blastocyst

The procedures used to produce in vitro fertilization (IVF) derived embryos are described in Appendix A. Cumulus oocyte complexes were allotted randomly to one of 5 treatments: 1) cultured continuously at 39°C for 24 h in OMM, 2) cultured at 41°C during the first 12 h of maturation (i.e., 0-12 h) followed by 39°C for the second 12 h of maturation (i.e., 12-24 h), 3) cultured at 39°C during 0-12 h followed by 41°C for 12-24 h, 4) cultured at 42°C during 0-12 h followed by 39°C for 12-24 h of maturation, and 5) cultured at 39°C during 0-12 h followed by 42°C for 12-24 h. After 24 h of culture, oocytes were washed in HEPES-TALP and then transferred to 600 µl IVF-TALP (n=25/well) containing epinephrine (2.5 µM), hypotaurine (10 µM) and penicillamine (20 µM) (Leibfried & Bavister, 1982). Spermatozoa were recovered from frozen/thawed semen from 3 bulls by Percoll gradient centrifugation (Appendix A) and added to IVF-TALP at an approximate concentration of 1 X 10^6 spermatozoa/mL. Eight hours after addition of sperm, putative zygotes were vortexed and washed extensively in HEPES-TALP to remove cumulus cells and associated spermatozoa. Zygotes/oocytes were then placed in CR1aa medium (10 zygotes/oocytes per 50 µl), covered with mineral oil, and

allowed to develop further. On d 5 post-IVF, 5 µl of neat htFCS was added to each microdrop. Number of embryos were recorded on d 3 and those that developed to blastocysts were recorded on d 7-9 after IVF.

Membrane Integrity

Cumulus oocyte complexes were randomly assigned to one of nine treatments to determine temperature effects on membrane integrity of oocytes in a 3 x 3 factorial with main effects of temperature (39, 41 or 42°C) and time (0-12, 12-24 or 0-24 h). Note that all oocytes for the treatment combinations 39°C/0-12, 12-24, 0-24 h received 24 h exposure to 39°C. However, oocytes were categorized into three separate groups to allow analysis of data using a factorial design. Following 24 h of culture, oocytes were denuded by vortexing in 50 µl HEPES-TALP for 7 min. Membrane integrity of oocytes was assessed using a dual staining procedure previously described by Takasaki (1971). Briefly, denuded oocytes were stained simultaneously with 0.01 M FDA and 0.1 M EtBr for 3 min. Fluorescence was viewed using an epifluorescence microscope and the number of green (FDA; membrane intact) and red/orange (EtBr; membrane permeable) oocytes were recorded. Treatments were replicated using a total of 33-69 oocytes per group.

Protein Synthesis

Cumulus oocyte complexes were cultured in either 1) CZB medium containing 10% htFCS and 50 µg/mL gentamicin or 2) OMM containing 10% htFCS instead of steer serum. Each 50 µl microdrop contained 50 µCi of a mixture of [^{35}S]methionine (70%) and [^{35}S]cysteine (15%). Cumulus oocyte complexes were cultured at 39°C continuously or at 41 or 42°C for 0-12 h or 12-24 h of maturation. Immediately following heat shock,

oocytes were denuded by vortexing as previously described, washed a minimum of 4 times in HEPES-TALP, checked microscopically to ensure oocytes were devoid of all cumulus cells, transferred in groups of ~15-25 to a minimum volume to 50 µl solubilization buffer [5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol] and frozen at -70°C until analysis. Incorporation of radiolabel into intracellular proteins of denuded oocytes was determined using TCA precipitation (Chapter 3).

Protein Synthesis as Modified by Cumulus Cells

A randomly chosen subset of COCs were denuded by vortexing. Denuded oocytes and remaining COCs were cultured at 39 or 42°C for 12 h in 50 µl CZB or OMM media containing 10% htFCS in microdrops with 50 µCi of a mixture of [^{35}S]methionine (70%) and [^{35}S]cysteine (15%). Afterwards, COCs were denuded. All oocytes were washed a minimum of 4 times in HEPES-TALP, checked microscopically to ensure absence of cumulus cells, transferred in a minimum volume to 50 µl solubilization buffer, and frozen in groups of ~15-25 at -70°C until analysis. Incorporation of radiolabel into intracellular proteins of denuded oocytes was determined by TCA-precipitation.

HSP70 Synthesis in Nonmatured and Matured Oocytes and 2-Cell Embryos

Oocytes were denuded by vortexing either immediately following collection (nonmatured) or after 24 h of culture in OMM at 39°C (matured). Two cell embryos were obtained by in vitro maturation, fertilization and culture (IVM/IVF/IVC) as previously described at 28-32 h post-insemination. Denuded oocytes/2-cell embryos were transferred to 50 µl of CZB medium containing 10% htFCS and 50 µCi of [^{35}S]methionine and [^{35}S]cysteine. Control oocytes and 2-cell embryos were incubated

with radiolabel at 39°C for 4 h and 20 min; heat-shocked groups were incubated with radiolabel at 42°C for 80 min followed by 39°C for 3 h. Oocytes and embryos were then washed 3 times in CZB + 0.1% (w/v) polyvinyl alcohol, transferred in a minimum volume to 50 µl solubilization buffer and frozen in groups at -70°C until analysis.

Incorporation of radiolabel into intracellular proteins was determined by TCA-precipitation. Proteins from solubilized oocytes and 2-cell embryos were analyzed using 2-D SDS-PAGE, with isoelectric focusing in the first dimension [1% (v/v) each of two preblended ampholines (pH 3.5-9.5 and pH 5.0-8.0)] and SDS-PAGE [10% (v/v) polyacrylamide] in the second dimension (Chapter 3). Radiolabeled proteins were visualized by fluorography using 1 M sodium salicylate as the flour. Each gel was loaded with 10,500-95,000 dpm of TCA-precipitable radioactivity and exposed to x-ray film; within each replicate, equal amounts of radioactivity were loaded for control and heat shock treatments and films were exposed for the same amount of time. The amount of radioactivity associated with each spot of interest was estimated indirectly by performing densitometric analysis (General Imaging, Microscan 1000 Gel Analysis System, Nashville, TN). The volume of each spot was calculated mathematically based on its area (size of spot) and height (intensity). Intensity of spots corresponding to three proteins (termed HSP68, P71 and P70) were reported in arbitrary units and expressed on a per embryo or oocyte basis. To validate the densitometric analysis, differing amounts of radioactive proteins were separated by one-dimensional SDS-PAGE and the volume of the most intense band was determined. There was a linear relationship between the amount of radioactivity loaded (R^2=0.93) and corresponding volume.

Statistical Analysis

Each experiment was replicated on several different days, using one or more microdrops of oocytes per treatment on each day. Data were analyzed by least-squares analysis of variance with the General Linear Models procedure of SAS (1989). Replicate (i.e., day) was used as the experimental unit for cleavage and development data because a single measurement was derived from examination of oocytes/embryos in one replicate. Data for cleavage and development within one replicate were analyzed two ways: with number cleaved or developing to the blastocyst stage as the dependent variable and total number of oocytes in the replicate as a covariate and 2) with percent cleaved or percent developed as the dependent variable. Method 1 was used to avoid problems of non-normality sometimes encountered with percentage data. The two analyses gave nearly identical results - for clarity, data are presented as percentages. However, probability values are derived from analysis of numbers cleaving or developing. Data on membrane integrity were analyzed similarly except that microdrop was the experimental unit because a single measurement was derived from examination of oocytes in one drop. Data for TCA-precipitable radioactivity were determined for a group of embryos from one microdrop and then mathematically expressed as dpm/oocyte or embryo. The experimental unit for densitometric analysis of HSP68, P70 and P71 was gel.

Each experiment was analyzed with all main effects [including treatment, stage of development, day (i.e., replicate) or other effects as appropriate for the experiment] and interactions in the model. All main effects were considered as fixed. For treatments and experiments with more than two levels, differences between levels were determined by orthogonal contrasts.

Results

Effects of Heat Shock during Oocyte Maturation on Cleavage Rate and Subsequent Development to Blastocyst

Exposure of oocytes to heat shock at specific times during maturation reduced the number of embryos cleaving and developing to the blastocyst stage (Table 4-1). Exposure to 41°C during the first or last 12 h of maturation did not affect cleavage rates as compared to oocytes cultured at 39°C. However, embryos formed from oocytes heat shocked at 41°C from 0-12 h of maturation were compromised in their ability to develop to the blastocyst stage ($P<0.02$). In contrast to the effects of 41°C, exposure of maturing oocytes to 42°C during the first or last 12 h of maturation greatly reduced the number of oocytes that cleaved and developed to the blastocyst stage ($P<0.001$; Table 4-1).

Membrane Integrity

Exposure of COCs to 41 or 42°C during the first or last 12 h of maturation or for the entire 24 h period did not alter membrane integrity of maturing oocytes. Membrane integrity for oocytes cultured at 39, 41 and 42°C was 95, 86 and 90 (SEM=3), respectively. Indeed, most oocytes exhibited intact membranes.

Protein Synthesis

Heat shock reduced the total amount of incorporation of radiolabel into intracellular proteins synthesized by maturing oocytes (Table 4-2; $P<0.003$). There was no temperature x time interaction, indicating that effects of temperature were similar during the first and last 12 h of culture. Amount of incorporation of radiolabel into proteins synthesized during the first 12 h of culture was similar to that incorporated during the last 12 h (24,657 and 23,025 dpm/oocyte, respectively; SEM=1804).

Table 4-1. Effects of heat shock on cleavage and development rates of COCs cultured at 39, 41 or 42°C during the first or last 12 h of maturation and then subsequently fertilized.

Temperature (°C)	Timing of heat shock (h)	No. oocytes	No. replicates[a]	% of Oocytes Cleaved[b]	% of Oocytes Developed[c]
39	0-24	121	3	81.1 ± 11.3	29.6 ± 1.2
41	0-12	147	3	71.4 ± 10.8	10.4 ± 1.1
41	12-24	147	3	74.5 ± 10.8	20.4 ± 1.1
42	0-12	141	3	35.7 ± 10.8	1.2 ± 1.1
42	12-24	130	3	15.9 ± 10.7	0.9 ± 1.1

[a] Number of days for which IVF was performed.

[b] Percentages of oocytes that had cleaved by d 3 post-IVF. Percentages were calculated for each replicate: data are least squares means ± SEM. Cleavage was affected by temperature ($P<0.03$); the 41 versus 42°C contrast was significant ($P<0.006$).

[c] Percentages of oocytes that developed to the blastocyst stage by d 7-9 post-IVF. Percentages were calculated for each replicate: data are least squares means ± SEM. Development was affected by temperature ($P<0.001$); the following orthogonal contrasts were significant: 39 versus 41 and 42°C ($P<0.002$), 41 versus 42°C ($P<0.001$) and 41°C/0-12 versus 41°C/12-24 h ($P<0.02$).

Table 4-2. Effects of culturing oocytes at 39, 41 or 42°C during the first or last 12 h of maturation on total incorporation of radiolabel into intracellular proteins.

Temperature (°C)	Timing of heat shock (h)	No. oocytes	No. replicates	TCA-precipitable radioactivity[a] (dpm/oocyte)
39	0-12	127	5	33,201
41	0-12	151	5	23,892
42	0-12	105	5	16,880
39	12-24	138	5	33,417
41	12-24	170	5	13,317
42	12-24	87	5	22,344

[a]Least squares means for TCA-precipitable radioactivity obtained from oocytes that had been cultured with cumulus cells in the presence of [^{35}S]methionine and [^{35}S]cysteine. Oocytes were denuded prior to analysis. Pooled SEM=3126. TCA-precipitable dpm was affected by temperature ($P<0.001$) but not by timing or temperature x timing. The orthogonal contrast of 39 vs 41 and 42°C was significant ($P<0.003$).

In the second experiment, the presence of cumulus cells influenced incorporation of radiolabel into proteins synthesized by maturing oocytes ($P<0.0001$) and altered subsequent responses to heat shock (temperature x cumulus, $P<0.0001$; Table 4-3). While the interaction reflects the fact that the absolute magnitude of decrease in TCA-precipitable radioactivity was greater for cumulus-intact cells, data suggest that removal of cumulus made cells more sensitive to heat shock. This is because heat shock reduced incorporation of radiolabel into proteins by 77% if oocytes were cultured without cumulus compared to 45% when oocytes were cultured while cumulus cells remained intact.

HSP70 Synthesis in Nonmatured and Matured Oocytes and 2-Cell Embryos

Representative fluorographs of proteins synthesized de novo by nonmatured and matured oocytes and 2-cell embryos radiolabeled at 39°C or while heat shocked are presented in Figure 4-1. Three different proteins believed to correspond to HSP70 were noted based on migration of proteins on 2-D gels as compared to HSP70 molecules in the literature. Two putative HSP70 molecules, designated P70 (M_r=69,680 $\pm$ 1796) and a slightly more basic P71 (M_r=70,991 $\pm$ 1779) were present in oocytes and two-cell embryos. The third protein identified, called HSP68, was of lower M_r (68,151 $\pm$ 1898) and more basic than P70 or P71 (Figure 4-1).

Equivalent amounts of P70, P71 and HSP68 were produced by nonmatured oocytes cultured at 39 or 42°C (Figure 4-1; Table 4-4). The same was true for matured oocytes except that P71 was absent. For 2-cell embryos, P70, P71 and slight amounts of HSP68 were present at 39°C. Exposure to 42°C greatly increased synthesis of HSP68

Table 4-3. Effects of heat shock and the presence of cumulus cells on incorporation of radiolabel into intracellular proteins synthesized by bovine oocytes.

	Temperature (°C)	No. oocytes	No. replicates	TCA-precipitable radioactivity ± SEM (dpm/oocyte)[a]
Cumulus intact	39	127	5	27,791 ± 813
	42	123	5	15,251 ± 814
No cumulus	39	90	5	5,155 ± 786
	42	89	5	1,182 ± 786

[a]Least squares means for TCA-precipitable radioactivity obtained from oocytes that had been cultured with cumulus cells intact or not in the presence of [^{35}S]methionine and [^{35}S]cysteine. Oocytes were denuded prior to analysis. TCA-precipitable radioactivity was affected by temperature ($P<0.0001$), cumulus cells ($P<0.0001$) and temperature x cumulus ($P<0.0001$).

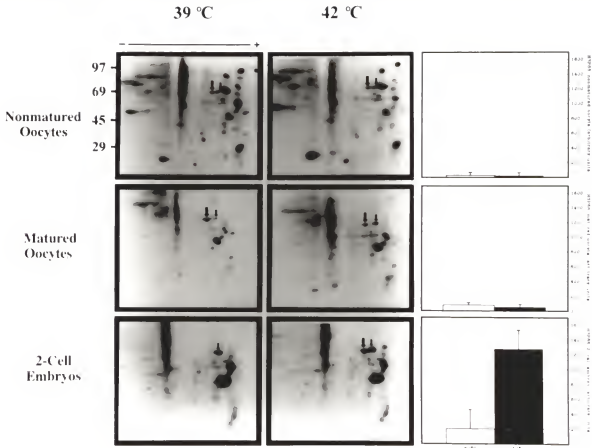

Figure 4-1. Representative profiles of [^{35}S]labeled proteins synthesized by nonmatured and matured oocytes as compared to IVF-derived 2-cell embryos cultured at 39 or 42°C and densitometric analysis of HSP68; proteins were assessed using 2-D SDS-PAGE and fluorography and densitometric analysis for HSP68 expressed on a per oocyte/embryo basis. Within a replicate, equal amounts of TCA-precipitable radioactivity were loaded per gel. Location of the region of the gel corresponding to P70 and/or P71 (small arrows) and HSP68 (large arrow) molecules is indicated by arrows. Note that P70 and P71 are apparent in nonnmatured oocytes and 2-cell embryos (small arrow located between the proteins) whereas P71 is absent in matured oocytes (small arrow pointing to P70).

Table 4-4. Effects of heat shock on de novo synthesis of P71, P71 and HSP68 by nonmatured and matured oocytes and 2-cell embryos as assessed by densitometric analysis[a].

Stage of development	No. oocytes	No. reps.	P71[b] 39°C	P71[b] 42°C	P70[c] 39°C	P70[c] 42°C	HSP68[d] 39°C	HSP68[d] 42°C
Nonmatured oocyte	80	2	46 ± 12	8 ± 12	664 ± 104	248 ± 104	26 ± 41	22 ± 41
Matured oocyte	102	4	ND	ND	19 ± 85	107 ± 84	78 ± 33	54 ± 33
2-cell embryo	214	8	391 ± 201	457 ± 201	801 ± 364	665 ± 364	198 ± 323	1274 ± 323

ND=not detectable.

[a] Results are least squares means of densitometric analysis (arbitrary units) ± SEM expressed on a per oocyte or embryo basis.

[b] Effects of temperature were nonsignificant ($P>0.10$).

[c] Effects of temperature were nonsignificant ($P>0.10$).

[d] Effect of temperature: nonmatured oocyte, $P>0.10$; matured oocyte, $P>0.10$; 2-cell embryo, $P<0.02$.

(Figure 4-1; Table 4-4) but had no consistent effect on synthesis of P70 and P71 (Table 4-4). Thus, heat-induced synthesis of HSP68 was noted as early as the 2-cell stage of development but not in unfertilized oocytes.

Discussion

The results of this study indicate that heat shock of oocytes can disrupt subsequent cleavage and development in a temperature- and stage-specific manner. Further, these effects are accompanied by an inhibition of protein synthesis that could be responsible for some of the adverse effects of heat shock. In contrast, there was no evidence that heat shock disrupts oocyte membrane integrity. Data also indicate that effects of heat shock on the oocyte may be reduced somewhat by the cumulus cells surrounding the oocyte. Finally, while the oocyte is incapable of increasing HSP70 synthesis in response to heat, this capability develops as early as the 2-cell stage. Thus, environmental signals are able to alter protein synthesis, and possibly gene expression, at a time several cleavage divisions before the commonly accepted timing of the maternal-zygotic transition in the bovine embryo.

The fact that exposure of bovine oocytes during maturation to elevated culture temperatures compromised subsequent cleavage and development indicates that effects of heat stress during the periestrual period on embryonic development (Putney et al., 1989a) may be due, at least in part, to direct effects of elevated maternal temperatures on the oocyte. Interestingly, exposure of oocytes to 41°C only compromised development of fertilized oocytes when given during the first 12 h of maturation. It is possible that disruption of events occurring during this time period are more likely to compromise oocyte potential for development than disruption of events occurring later. Key events

occurring during the first 12 h of maturation include posttranslational modification of cyclin dependent proteins (Wickramasinghe & Albertini, 1993), rearrangement of cytoskeletal framework necessary for breakdown of the germinal vesicle and progression to metaphase I (Sirard et al., 1989). In the mouse, heat shock disrupted spindle formation during metaphase I and reduced ability to progress to metaphase II (Baumgartner & Chrisman, 1987).

Requirement of companion cumulus cells during the growth and maturation of the female gamete has been well established (Buccione et al., 1990) and present findings indicate that an oocyte's ability to incorporate radiolabel into proteins is compromised upon loss of cumulus. Moreover, cumulus cells may have ameliorated partially the inhibitory effects of heat shock on incorporation of radiolabel into newly synthesized proteins. This effect is somewhat difficult to interpret since removal of cumulus cells reduced incorporation of radiolabel into proteins by oocytes at 39 and 42°C. Nonetheless, the percent reduction in incorporation caused by heat shock was greater if cumulus were not present. Since cumulus cells and the oocyte interact through gap junctions (Buccione et al., 1990), it is possible that small thermoprotective molecules such as glutathione (Aréchiga et al., 1995) are transferred from the cumulus to the oocyte. Alternatively, cumulus may provide extracellular thermoprotectants or produce regulatory molecules that cause activation of thermoprotective mechanisms within the oocyte. If cumulus are important to the thermal resistance of oocytes, it is pertinent to note that cumulus cells are lost soon after ovulation in species such as the bovine (Lorton & First, 1979). Perhaps thermoprotectants in oviductal fluid such as taurine (Fahning et al., 1967) aid in protecting the oocyte once it enters the oviduct.

Exposure of 2-cell embryos to heat shock resulted in a dramatic increase of a protein designated as HSP68 because of its similarity in molecular weight and isoelectric point to a heat-inducible protein previously characterized in other cells (Hunt & Calderwood, 1990; Guerriero et al., 1989) and because of its heat inducibility. Two other proteins that may be members of the HSP70 family based on their migration in 2-D gels did not appear to be heat inducible and were not designated as heat shock proteins. The fact that heat shock caused a large increase in the synthesis of HSP68 at the 2-cell stage is remarkable because this stage is earlier than the reported stage at which the embryonic genome is activated in the bovine [8-16-cell] (Camous et al., 1986; King et al., 1988; Frie et al., 1989). The heat-induced increase of HSP68 in the 2-cell embryo is due to either 1) increased stability of maternal HSP68 mRNA, 2) increased translational efficiency of maternal HSP68 mRNA or 3) increased transcription of the embryonic HSP68 gene. Of these possibilities, increased stability of maternal HSP68 mRNA is the least likely. Maternal pools of mRNA decrease dramatically during oocyte maturation and are at very low levels by the 2-cell stage of development (Paynton et al., 1988). In the mouse, most maternal HSP70 mRNA is depleted before zygotic activation at the 2-cell stage (Manejwala et al., 1991). If the primary effect of heat shock was to increase mRNA stability of HSP68 in the 2-cell embryo, one would also expect heat-induced increases of this protein in oocytes. This was not noted, however. Increased translational efficiency of maternal HSP68 mRNA remains a possibility. For most cell types studied thus far, regulation of HSP70 synthesis is at the level of transcription (Tanguay, 1988; Nover & Scharf, 1991; Morimoto, 1993; Ezzell, 1995). It is possible, therefore, that as for other cells, increased HSP68 synthesis in 2-cell embryos is a consequence of transcriptional

activation of the HSP68 gene expression (Morimoto, 1993). The observation that P71 was present in nonmatured oocytes, undetectable after 24 h of culture, and then reappeared in the 2-cell embryo provides further support that 2-cell embryos are transcriptionally competent. In any case, results indicate that the bovine embryo can respond to changes in its environment very early in development by modifying synthesis of specific proteins.

While the 8-16-cell stage is usually the time when the maternal-zygotic transition is thought to occur (Camous et al., 1986; King et al., 1988; Frie et al., 1989), the suggestion that IVF-derived embryos are transcriptionally active prior to this stage is not new. Changes in gene expression as assessed using [^{3}H]uridine incorporation (Plante et al., 1994) and 2-D SDS-PAGE analysis of radiolabeled proteins (Barnes & First, 1991) have been reported as early as the 2 to 4-cell stage of development. Culture may also modulate gene expression. Vernet et al. (1993) reported that the transcriptional activity of early mouse embryos was affected by their manipulation and culture. Moreover, expression of HSP70.1 was much higher in embryos that developed in culture when compared to those developed in vivo (Christians et al., 1995). Furthermore, Ealy and Hansen (1994) demonstrated that timing of induced thermotolerance (a phenomenon whereby exposure to a mild heat shock confers thermotolerance to a subsequent more severe heat shock) differs for mouse embryos that developed in culture as compared to those that developed in vivo.

The role for HSP70 molecules in developmental acquisition of thermotolerance in the early embryo is unclear. Early data were suggestive that acquisition of thermotolerance may be related to an ability to produce HSP70 molecules (Morange et

al., 1984; Hahnel et al., 1986). Hendrey and Kola (1991) demonstrated that microinjection of HSP70 mRNA conferred thermal resistance in murine oocytes when exposed to 42-43°C. However, more recent data indicates that heat shock induced synthesis of HSP70 can precede induced thermotolerance (Chapter 3) and thus other factors in addition to HSP70 are involved with thermotolerance.

In conclusion, thermolability of bovine oocytes is most prominent during the first 12 h of maturation and may be due to alterations in protein synthesis and absence of heat inducibility of heat shock proteins. Data also suggest a possible thermoprotective role for cumulus cells during oocyte maturation. Perhaps most interestingly, results indicate that the 2-cell embryo is capable of altering protein synthesis and possibly gene expression, in face of a hostile environment.

CHAPTER 5
ONTOGENY OF TEMPERATURE-REGULATED HEAT SHOCK PROTEIN 70 SYNTHESIS IN PREIMPLANTATION BOVINE EMBRYOS DERIVED BY IN VITRO FERTILIZATION

Introduction

Early cleavage stage bovine embryos must rely on maternal pools of mRNA stored during oogenesis for synthesis of proteins. Activation of the embryonic genome in large part does not occur until the 8 to 16-cell stage (Camous et al., 1986; King et al., 1988; Frei et al., 1989). Incubation of embryos with transcription inhibitors prior to this stage does not block subsequent development until the 8 to 16-cell stage (Barnes & First, 1991). Further analysis utilizing [^{3}H]uridine incorporation and α-amanitin sensitive protein synthesis suggest that partial genome activation may occur as early as the late 2-cell to 4-cell stage of development (Barnes & First, 1991; Plante et al., 1994; Marcucio et al., 1995). Exposure of embryos to elevated temperature at the 2-cell stage and 4 to 8-cell stages blocks subsequent development (Ealy et al., 1995; Chapter 6). In contrast, morulae, which have already undergone embryonic genome activation, are more resistant to elevations in temperature (Ealy et al. 1995; Chapter 6). Thus, the early embryo may be sensitive to noxious environmental stimuli because it is transcriptionally quiescent and has limited capacity to respond to changes in its environment. Yet, recent data indicates that 2-cell embryos can respond to elevated temperature by increasing synthesis of a heat-inducible form of heat shock protein 70 called HSP68 (Chapter 4).

The observation that heat shock induced HSP68 in 2-cell embryos was remarkable because it suggested that environmental signals can activate certain genes prior to embryonic genome activation. Members of the HSP70 family have been implicated in cellular thermotolerance of a variety of cell types including fibroblasts (Riabowol et al. 1988) and oocytes (Hendrey & Kola, 1991). These proteins are believed to provide thermoprotection by refolding damaged proteins and protecting ribosomal RNA (Duncan & Hershey, 1989; Nover & Scharf, 1991).

In many cells, heat-induction of HSP70 synthesis is regulated at the level of transcription requiring interaction of a HSF with the promoter region of the HSP70 gene (Nover & Scharf, 1991; Morimoto, 1993). In the mouse embryo, binding of HSF to HSE has been reported to occur at all stages of development except for the 4-cell (Mezger et al., 1994). However, post-transcriptional regulation of HSP70 synthesis has been reported in other cell types (Banerji et al., 1984; Theodorakis et al., 1988). Heat-inducibility of HSP68 synthesis in the bovine 2-cell embryo may represent either alterations in the transcriptional competency of early embryos, increased mRNA stability or increased translation. Objectives were to determine the ontogeny of synthesis of members of the HSP70 family in preimplantation bovine embryos and to ascertain whether heat-induced increases in HSP68 in 2-cell embryos are due to environmental alterations in gene expression or to posttranscriptional regulation.

Materials and Methods

Materials

Aprotinin, α-amanitin, control mouse ascites fluid, estradiol, heparin, HEPES, leupeptin, mineral oil, pepstatin, polyvinyl alcohol, sodium pyruvate, antibiotics and

Tissue Culture Medium-199 (TCM-199) were purchased from Sigma Chemical Company (St. Louis, MO). AEBSF (4-(2-Aminoethyl)-benzenesulfonylfluoride, hydrochloride) was purchased from Boehringer Mannheim (Indianapolis, IN). A mouse monoclonal antibody (IgG, clone BB70) raised against chicken HSP70 and specific for both constitutive and inducible forms of HSP70 was purchased from StressGen (Victoria, BC, Canada). Protein G coupled to agarose was purchased from Oncogene Science (Cambridge, MA). Bovine steer serum and fetal calf serum were purchased from Pel-Freez (Rogers, AK) and Atlanta Biologicals (Norcross, GA), respectively. Frozen semen from various bulls was obtained from American Breeders Service (Madison, WI) or was produced locally. K562 cells were obtained from American Type Culture Collection (Rockville, MD).

Materials purchased from Fisher Scientific (Fair Lawn, NJ) included acrylamide solution (40%), ethidium bromide (EtBr), sodium salicylate and trichloroacetic acid. X-ray film was from Fuji (Tokyo, Japan) or Kodak (Rochester, NY). Radiolabeled [^{35}S]methionine and [^{35}S]cysteine (70:15% mixture, respectively; specific activity > 1000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL) or ICN (Costa Mesa, CA). Ampholines were purchased from Pharmacia (Uppsala, Sweden).

The CZB medium was prepared as described by Chatot et al. (1989). Modified Tyrode's solutions (HEPES-TALP, IVF-TALP and SP-TALP; Parrish et al., 1986) were prepared in the laboratory or were purchased from Specialty Media, Inc. (Lavallette, NJ). The CR1aa medium was prepared as described by Rosenkrans et al. (1993). Sterile saline [0.9% (wt/vol) NaCl] was supplemented with 550 µg/L amphotericin, 100,000 IU/L penicillin-G and 100 mg/L streptomycin (ABAM) or with 100,000 IU/L Penicillin-

G and 100 mg/L streptomycin (Pen/Strep; Sigma Chemical). Pituitary-derived follicle stimulating hormone (FSH-P) was purchased from Schering (Kenilworth, NJ).

In Vitro Maturation, Fertilization and Culture of Embryos

The procedures used to produce embryos were as described in Appendix A. Briefly, oocytes were matured for 24 h and then fertilized with Percoll-purified sperm for 16 to 20 h. Putative zygotes were then vortexed and washed extensively in HEPES-TALP to remove cumulus cells and associated spermatozoa. Putative zygotes were either cocultured with oviductal cells (10-15 oviductal worms/50 µl) in LEC [TCM-199 with Earle's salts, 10% (v/v) steer serum, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate; 20 zygotes/oocytes per 50 µl microdrop) or CR1aa (10 zygotes/oocytes in 50 µl microdrop) covered with mineral oil and allowed to develop further. When LEC was used as the culture medium, an additional 50 µl was added to each microdrop on d 3 post-IVF. Neat htFCS (5 µl) was added to culture drops on d 5 post-IVF when CR1aa was used. Embryos at the 2-cell, 4-cell, 8-cell, 16-32-cell, morula and blastocyst stages were obtained on d 1, 2, 3, 4, 5 and 7-9 post-IVF, respectively.

HSP70 Synthesis in Preimplantation Embryos

At the desired stage of development, embryos were removed from culture drops and washed once in HEPES-TALP and then transferred in groups of 5 to 10 to 50 µl microdrops of CZB medium containing 10% FCS and 50 µCi L-[^{35}S]methionine and L-[^{35}S]cysteine. Control embryos were incubated with radiolabel at 39°C for 4 h and 20 min; heat-shocked groups were incubated with radiolabel at 41 or 42°C for 80 min followed by 39°C for 3 h. Embryos were then washed 3 times in CZB + 0.1% (w/v) polyvinyl alcohol, transferred in a minimum volume to 50 µl 5 mM K_2CO_3 containing 9.4

M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol and frozen at -70°C until analysis.

Incorporation of radiolabel into intracellular proteins was determined by TCA precipitation (Mans and Novelli, 1961). Proteins from solubilized embryos were analyzed using 2-D SDS-PAGE as previously described (Chapter 3). Each gel was loaded with 10,500-95,000 dpm of TCA-precipitable radioactivity and exposed to x-Ray film; within each replicate, equal amounts of radioactivity were loaded for control and heat shock treatments and films were exposed for the same amount of time. The amount of radioactivity associated with each spot of interest was estimated indirectly by performing densitometric analysis (General Imaging; Microscan 1000 Gel Analysis System, Nashville, TN). The volume of each spot was calculated mathematically based on its area (size of spot) and height (intensity). Intensity of spots corresponding to HSP68, HSC71 and HSC70 were expressed on a per embryo basis in arbitrary units. Validation of densitometric analysis was previously described (Chapter 4).

Immunoprecipitation of HSP70 Molecules

Expanded blastocysts were washed once in HEPES-TALP and then transferred in groups of 5 to 15 to 50 µl of CZB medium containing 10% FCS and 50 µCi of L-[^{35}S]methionine and L-[^{35}S]cysteine. Control embryos (n=95) were incubated with radiolabel at 39°C for 5 h; heat-shocked embryos (n=95) were incubated with radiolabel at 43°C for 2 h followed by 39°C for 3 h. Embryos were solubilized by sonication for 10-20 sec in 300 µl of RIPA buffer that consisted of 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate and 0.1% (w/v) SDS solution containing 4 mM AEBSF, 1 µg/mL aprotinin and 10 µg/mL leupeptin. Cell lysates of pooled control and heat

shocked embryos were precleared by incubating with 50 µl control mouse ascites fluid (CMAF) and 25 µl Protein G agarose for 1 h at 4°C. Protein G agarose was pelleted by centrifugation and the supernatant fraction was collected. Supernatant fractions were divided equally and incubated with either HSP70 antibody (10 µg) or CMAF (10 µg) and with Protein G agarose (25 µl) overnight at 4°C on a tube rotator. Total amount of TCA-precipitable radioactivity used in immunoprecipitation reaction was 4.4 and 6.3 million dpm for control and heat shocked embryos, respectively. Immunoabsorbed Protein G complexes were then collected using low speed centrifugation (~2500 rpm) for 15 min at 4°C and were washed 4 X in RIPA supplemented with protease inhibitors. After the final wash, the pellet was resuspended in 40 µl dH_2O and boiled for 5 min. Samples were centrifuged for 5 min at 2500 rpm and the supernatant fraction was vacuum lyophilized, resolubilized in 50 µl 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol, and analyzed by 2-D SDS-PAGE and fluorography. To verify recovery of antigen/antibody complex from Protein G agarose, 25 µl of 5 mM Tris-HCL (pH=6.8), 15% (w/v) glycerol, 5% (w/v) SDS and 5% (w/v) β-mercaptoethanol was added to the remaining pellet and boiled for 5 min. Immunoprecipitates were analyzed by 1-D SDS-PAGE using 12.5% polyacrylamide and fluorography with 1 M sodium salicylate as the flour. The experiment was replicated twice.

Culture and Radiolabeling of Embryos with α-amanitin

Putative zygotes were randomly placed in culture medium (either LEC that had been preconditioned with oviductal cells or CR1aa medium) containing 0 or 50-100 µg/mL α-amanitin at 10-18 hpi. Microdrops were then examined at 28-29 hpi; all 2-cell, 4 to 8-cell embryos present were removed from culture, sorted according to stage of

development, washed once in HEPES-TALP and then equally and randomly assigned within α-amanitin groups to heat shock treatment. Additionally a replicate of 8-cell embryos that received α-amanitin beginning at 10-18 hpi was collected at 48 hpi. Also, blastocysts collected on d 7 post insemination were cultured with α-amanitin (50-100 μg/mL) for 6 h before radiolabeling. At all stages, control embryos were radiolabeled at 39°C continuously in the presence or absence of α-amanitin; heat shocked embryos were radiolabeled in the presence or absence of α-amanitin at 42°C for 80 min followed by 39°C for 3 h. Analysis of effects of heat shock and α-amanitin on HSP68 synthesis were evaluated using 2-D SDS-PAGE and fluorography as described earlier.

Biological activity of α-amanitin was determined by incubating K562 cells with 0 or 50 μg/mL α-amanitin and 1.25 μCi/μl of [^{35}S]methionine and [^{35}S]cysteine for 4 h 20 min. Addition of α-amanitin decreased total amount of TCA-precipitable radioactivity by 74.6% (16,367 ± 288 versus 4158 ± 78 for control and treated cells, respectively).

Statistical Analysis

Each experiment was performed on several different days, using one or more gels (replicates) of embryos per treatment on each day. Data were analyzed using least squares analysis of variance using the General Linear Models procedure of SAS (1989). TCA-precipitable radioactivity estimated within a given microdrop was expressed on a per embryo basis. Similarly, the peak volume obtained from densitometric analysis for HSP68, HSC71 and HSC70 was also expressed on a per embryo basis. Statistical models included sources of variation due to stage of development, temperature and replicate. When applicable, effects of α-amanitin were included in the model. Because of heterogeneity of variance, data were log-transformed before analysis. Least squares

means presented represent values calculated from analyses derived from within a stage of development and without transformation. Probability values are those derived from analysis of log-transformed data.

Results

Identification and Characterization of HSP70 Synthesis

Representative fluorographs of proteins synthesized de novo by blastocysts cultured and radiolabeled at 39, 41 or 42°C are presented in Figures 5-1 and 5-2. At all stages of development, three different proteins were identified that were of the same approximate M_r and isoelectric point as the heat-inducible HSP68 and the putative heat shock proteins called P71 and P70 that were previously identified in the bovine 2-cell embryo (Chapter 4; Figure 4-1). This was true whether embryos were radiolabeled with ^{35}S or ^{3}H at 41°C (Figure 5-3). Radiolabeled proteins corresponding to these proteins could be specifically immunoprecipitated by an antibody that recognizes the constitutive and inducible forms of HSP70 (Figure 5-2). Therefore, based on size and relative degree of heat-inducibility, P71 and P70 were designated as HSC71 and HSC70 and the identity of the protein called HSP68 was confirmed. Visual inspection of fluorographs of radiolabeled proteins indicated that HSC71 and HSC70 were constitutively synthesized by all embryos. The third protein, HSP68 was either absent or synthesized in low amounts in embryos cultured at 39°C. Exposure of embryos to 42°C greatly increased the synthesis of this protein. One to three isoelectric variants of this protein were noted (Figures 5-1 and 5-3). In contrast, no consistent effects of heat shock on synthesis of HSC71 and HSC70 were noted until the expanded blastocyst stage. Addition of htFCS to medium was not required for the synthesis of heat-induced or cognate HSP70 molecules (data not shown).

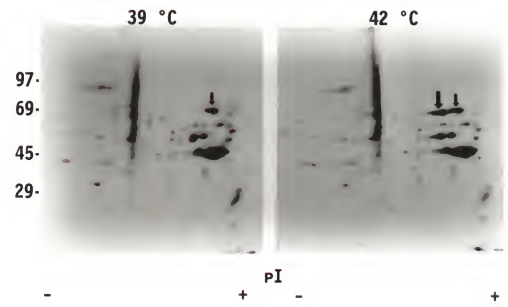

Figure 5-1. Representative fluorographs of [^{35}S]methionine and [^{35}S]cysteine labeled proteins synthesized de novo by bovine expanded blastocysts cultured at either 39 or 42°C. Each gel was loaded with an equivalent amount of TCA-precipitable radioactivity (50,000 dpm) corresponding to 0.2 and 0.6 embryos/gel for 39 and 42°C. All embryos, regardless of treatment, synthesized two cognate HSP70 molecules referred to as HSC71 and HSC70 (small arrows). A third protein was identified when embryos were cultured at 42°C that was of lower M_r and slightly more basic and was referred to as HSP68 (large arrow).

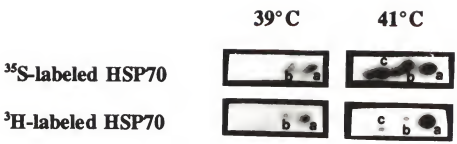

Figure 5-2. Representative fluorographs of [^{35}S]methionine and [^{35}S]cysteine labeled proteins or [^{3}H]-leucine labeled proteins synthesized de novo by compacted morula at 39 or 41°C. Each gel was loaded with an equivalent amount of TCA-precipitable radioactivity (25,000 dpm) corresponding to 2.8 and 1.1 embryos/gel for 39 and 41°C. Only the portion of the fluorographs corresponding to HSP70 is shown.

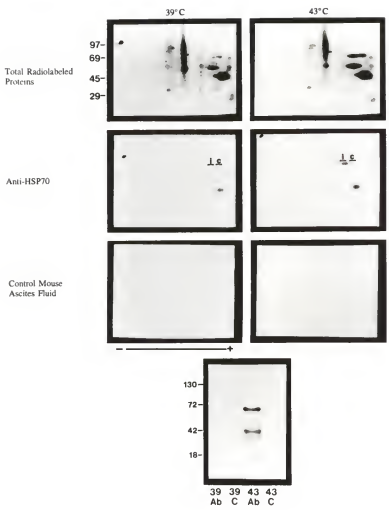

Figure 5-3. Immunoprecipitation of embryonic HSP70. Embryos were cultured in the presence of [^{35}S]methionine and [^{35}S]cysteine at 39 (n=95) or 43°C (n=95). Sonicated cell lysates of control and heat shocked embryos were incubated with either HSP70 antibody or CMAF along with Protein G agarose. Total radiolabeled proteins were analyzed using 2-D SDS-PAGE and fluorography by loading an equivalent amount of TCA-precipitable radioactivity (75,000 dpm) per gel (top panel). Immunoprecipitates were analyzed by 2-D SDS-PAGE and fluorography by loading an equivalent amount embryos per gel (middle panel). In addition, residual antigen/antibody complexes bound to Protein G agarose were stripped with additional boiling and analyzed by 1-D SDS-PAGE and fluorography (bottom panel). Note that anti-HSP70 precipitated 3 isoforms of a heat-inducible (i) protein, termed HSP68 and 2 cognate proteins (c) termed HSC71 and HSC70. All of these proteins were observed when embryos were cultured at 43°C but only HSC70 and slight amounts of HSP68 were detectable at 39°C. A fourth protein was also noted corresponding to 42 kDa that is likely to be an actin that precipitated as part of an actin-HSP70 complex.

HSP70 Synthesis at Different Stages of Development

Effects of heat shock at different stages of development on synthesis of HSP68, HSC71, HSC70 and total intracellular protein are presented in Figure 5-4. Exposure of preimplantation bovine embryos to 42°C resulted in a dramatic increase in the amount of HSP68 that was synthesized per embryo (temperature $P<0.01$). Numerical increases of HSP68 were noted in 37 of the total 40 replicates of embryos examined. In contrast, HSC71 and HSC70 synthesis were not consistently altered by heat shock until the expanded blastocyst stage. Exposure of expanded blastocysts to heat shock not only increased HSP68 synthesis but increased the total amount of HSC71 (5079 versus 9486; SEM=966; $P<0.07$) and HSC70 (7408 versus 19,751; SEM=1871; $P<0.02$) synthesized per embryo.

Overall, heat shock did not alter the total amount of incorporation of radiolabel into intracellular proteins synthesized per embryo; incorporation was dependent on stage of development ($P<0.0001$). Total amount of incorporation of radiolabel into intracellular proteins synthesized by 4-cell, 8-cell, 16-32-cell and morula stage embryos was similar. Thereafter, incorporation of radiolabel into proteins increased approximately 6-fold by the blastocyst stage and continued to increase following expansion and hatching. The absence of an effect of heat shock on reducing incorporation of radiolabel into intracellular proteins in cleavage stage embryos was in part due to a high degree of heterogeneity of variance. When effects of heat shock were determined comparing responses of 2-cell and blastocyst stage embryos in a separate data set, exposure of 2-cell embryos to 42°C decreased ($P<0.01$; 8038 versus 4786;

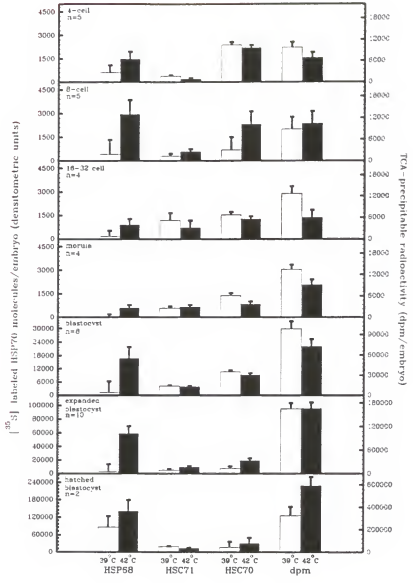

Figure 5-4. Effects of exposure of preimplantation embryos to 42°C on synthesis of HSP68, HSC71 and HSC70 (densitometric analysis) and total intracellular protein synthesis (TCA-precipitable radioactivity/embryo). Within replicate, each gel was loaded with an equivalent amount of TCA-precipitable radioactivity (10,000 up to 95,0000 dpm) and x-ray film was exposed for the same amount of time (n=number of replicates/stage). *Note that no conclusions regarding stage of development differences in the absolute magnitude of HSP70 molecules can be derived because embryos were not analyzed contemporaneously and there were differences between replicates in the amount of time the x-ray film was exposed.* Data are least squares means ± SEM computed within each stage of development.

SEM=393) incorporation of radiolabel into intracellular protein but did not alter protein synthetic capabilities of expanded blastocysts (165,311 versus 166,284; SEM=14473).

Culture and Radiolabeling of Embryos with α-amanitin

Effects of heat shock and incubation with α-amanitin on 2-cell embryos are presented in Figure 5-5. Exposure to 42°C ($P<0.01$) and α-amanitin ($P<0.01$) decreased the total amount of incorporation of radiolabel into intracellular proteins synthesized during the experimental period. Heat shock increased ($P<0.001$) the synthesis of HSP68 and addition of α-amanitin (a potent inhibitor of RNA polymerase II) did not block this heat-induced increase. There were no effects of heat shock or α-amanitin on synthesis of HSC71 or HSC70.

Results comparing responses of 2-cell, 4-cell, 4 to 8-cell, 8-cell and blastocysts cultured at 39 or 42°C in the presence or absence of α-amanitin are presented in Figure 5-6. Synthesis of HSP68 was increased by 42°C at all stages examined ($P<0.09$). When all embryos at the 4-cell stage or greater were analyzed together, treatment with α-amanitin inhibited heat-induced increases of HSP68 in embryos (temperature x α-amanitin; $P<0.08$). Note that the 4-cell and 4- to 8-cell embryos were collected at 28-29 hpi and were thus of equivalent age to the 2-cell embryos in Figure 5-5. There were no effects of heat shock or α-amanitin on synthesis of HSC71 or HSC70 (results not shown).

Discussion

Bovine embryos during the early cleavage period proceed under the direction of maternal mRNA. An inactive genome prior to the 8 to 16-cell (Camous et al., 1986; King et al., 1988; Frei et al., 1989) or late 4-cell stage (Barnes & First, 1991) suggests that bovine embryos would have limited ability to respond to changes in their

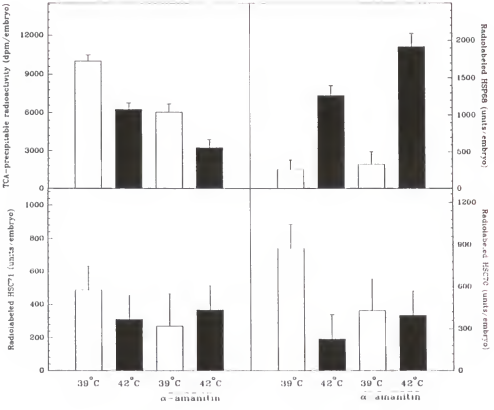

Figure 5-5. Effects of heat shock and α-amanitin on total TCA-precipitable radioactivity and synthesis of HSP70 molecules in 2-cell embryos. Embryos were cultured in the presence or absence of α-amanitin with [^{35}S]methionine and [^{35}S]cysteine at 39 or 42°C. Groups of embryos (3-22) were analyzed by 2-D SDS-PAGE, fluorography and densitometry. For each replicate (n=6), the amount of radioactivity loaded per gel and exposure times for x-ray film were identical for 39 and 42°C groups. Data are least squares means ± SEM.

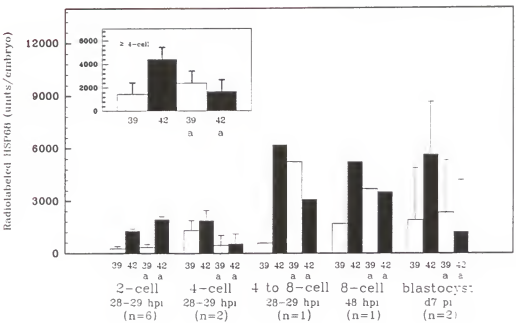

Figure 5-6. Effects of heat shock (42°C) and α-amanitin (a) on total TCA-precipitable radioactivity and HSP68 synthesis in bovine embryos. Embryos were cultured in the presence of [^{35}S]methionine and [^{35}S]cysteine at 39 or 42°C. Groups of embryos (3-22) were analyzed by 2-D SDS-PAGE and fluorography. For each replicate, the amount of radioactivity loaded per gel and exposure times for x-ray film were identical for 39 and 42°C groups. Data are least squares means ± SEM. Data obtained from 2-cell embryos were previously illustrated in Figure 5-5 and are included in this figure for comparison to later stages of development. When embryos at the 4-cell stage or greater were analyzed together, treatment with α-amanitin inhibited heat-induced increases in HSP68 in embryos (inset, $p < 0.08$).

environment. Yet, present data indicate that bovine embryos can respond to a hostile thermal environment by synthesizing increased amounts of HSP68 as early as the 2-cell stage (Chapter 4, present study). The mechanism for this increase in HSP68 synthesis is dependent upon stage of development. Clearly by the 4-cell stage, heat-induced synthesis of HSP68 is regulated at the level of transcription because addition of α-amanitin blocked heat-induced synthesis of HSP68 at the 4-cell and all subsequent stages of development examined. Transcriptional regulation of heat-inducible HSP70 in most cells is a result of trimerized HSFs binding to HSEs in the promoter region of the HSP70 gene (Morimoto, 1993). In contrast, α-amanitin did not block heat-induced synthesis of HSP68 in 2-cell embryos, suggesting that regulation of HSP68 by heat shock at this stage is caused by increased maternal mRNA stability or translational efficiency. Of these possibilities increased stability of maternal HSP68 mRNA is the least likely. In the mouse, there is a precipitous decline in the maternal pool of mRNA following resumption of meiosis such that levels are very low by the 2-cell stage (Paynton et al., 1988). Moreover, most HSP70 mRNA is depleted before activation of the embryonic genome in 2-cell mouse embryos (Manejwala et al., 1991). If the primary effect of heat shock was to increase mRNA stability of HSP68 in the bovine 2-cell embryo, one would also expect heat-induced increases of this protein in oocytes. This was not noted, however (Chapter 4). Preferential translation of HSP68 mRNA is more probable because this phenomenon has been reported for other heat shock proteins (Colbert & Young, 1987). In chicken reticulocytes, hsp70 mRNA is moderately abundant. Increased synthesis of HSP70 following heat shock was independent of changes in hsp70 mRNA and occurred in the presence of actinomycin-D (Banerji et al., 1984; Theodorakis et al., 1988).

Heat-induced transcriptional activation at the 2-cell stage of development cannot be ruled out entirely, however. In *Drosophila*, hsp70 gene expression is regulated at the level of elongation rather than binding of RNA polymerase II to the promoter (Gilmour & Lis, 1986). RNA polymerase II is engaged but paused in the promoter region prior to activation of transcription; binding of HSF allows for elongation of transcript. If such a mechanism is operational in 2-cell embryos, it is likely to be insensitive to α-amanitin because this inhibitor acts to block transcription by preventing RNA polymerase II from binding the promoter region of the gene (Wieland & Faulstich, 1978).

Embryos at the 2-cell, 4-cell and 4 to 8-cell stage were all collected at 28-29 hpi. Thus, differences between 2-cell embryos as compared to embryos at the 4- and 8-cell stages reflect cleavage dependent mechanisms. Constitutive expression of HSP70.1 during genome activation in the mouse (Christians et al., 1995) is dependent upon progressive maturation of chromatin structure (Thompson et al., 1995).

Gene expression using in vitro derived embryos may have been modulated by culture conditions. Vernet et al. (1993) reported that the transcriptional activity of early mouse embryos was affected by their manipulation and culture. Similarly, Ealy and Hansen (1994) demonstrated that timing of induced thermotolerance in mouse embryos occurs earlier at an earlier developmental stage for embryos that developed in vitro as compared to those allowed to develop in vivo. Moreover, expression of HSP70.1 was much higher in embryos developed in culture developed when compared to those developed in vivo (Christians et al., 1995).

Bovine embryos become more resistant to heat shock as they proceed in development: deleterious effects of heat shock are more prominent in 2-cell embryos than

morula (Ealy et al., 1995; Chapter 6). In the present study, exposure of 2-cell embryos to 42°C decreased incorporation of radiolabel into total intracellular proteins but did not alter protein synthetic capabilities of expanded blastocysts. Exposure of hatched blastocysts to heat shock increased incorporation of radiolabel into proteins. Such a positive effect of heat shock on protein synthesis might be expected in a thermotolerant cell because elevated temperature increases chemical reaction rates in the cell. Similar positive effects of heat shock on incorporation of radiolabel into proteins have been observed for oviductal and endometrial tissue (Malayer & Hansen, 1990). Thus, data on incorporation of radiolabel into proteins are a further demonstration that embryonic resistance to heat shock increasing during development.

Differences in thermal sensitivity to heat shock between 2-cell embryos versus other stages are not caused by an inability of 2-cell embryos to synthesize heat shock proteins. Heat-induced synthesis of HSP68 and constitutive synthesis of HSC71 and HSC70 were noted at every stage of development examined. Heat-inducibility of HSC71 and HSC70 was noted only in expanded blastocysts and, therefore, some of the resistance of later stage embryos to heat shock might be caused by changes in regulation of constitutive HSP70 molecules. It is also possible that quantitative differences in the increases of HSP68 exist during development or that other mechanisms in addition to HSP70 are involved in developmental acquisition of thermotolerance. One candidate is the antioxidant, GSH. Intracellular stores are relatively high in the mouse oocyte and decrease following resumption of meiosis (Gardiner & Reed, 1994). The precipitous decline in GSH is coincident with increased thermal sensitivity of the 2-cell embryo. Ability to synthesize increased amounts of GSH is not noted in the mouse until the

blastocyst stage (Gardiner & Reed, 1995). In many cells heat shock increases free radical production (Loven, 1988) and causes a decrease in GSH (Aréchiga et al., 1995). Addition of GSH increased survival of bovine embryos following heat shock (Ealy et al., 1992). Also, BSO, an inhibitor of glutathione synthesis, prevented induced thermotolerance in murine morula (Aréchiga et al., 1995). Harris et al. (1991) demonstrated that BSO attenuated the thermotolerance response in postimplantation rat embryos without decreasing HSP70 mRNA or HSP70 synthesis.

In conclusion, environmental signals can activate gene expression before EGA usually occurs in bovine embryos. This observation is remarkable because it confirms that early embryos are not transcriptionally quiescent but capable of responding to heat shock by increasing the synthesis of HSP68. Developmental acquisition of thermotolerance was not related to an ability to synthesize HSP70, suggesting the involvement of other intracellular systems in this process.

CHAPTER 6
DIFFERENTIAL RESPONSES OF BOVINE OOCYTES AND PREIMPLANTATION EMBRYOS TO HEAT SHOCK

Introduction

It is well documented that mammalian females exposed to heat stress experience increased embryonic mortality (Thatcher & Hansen, 1993). Effects of maternal hyperthermia are most pronounced when experienced between the onset of estrus and insemination (Putney et al., 1989a) and during the first few cleavage divisions of the early embryo (Dutt, 1963; Tompkins et al., 1967; Putney et al., 1988a; Ealy et al., 1993). Effects of heat stress decline as pregnancy proceeds such that effects are minimal by d 3 to 5 of pregnancy in the ewe (Dutt, 1963) and cow (Ealy et al., 1993) and by d 5 in the pig (Tompkins et al., 1967). Increased embryonic mortality associated with maternal hyperthermia is in part mediated by direct effects of elevated temperature on gametes and early embryos because exposure of embryos to increased culture temperature decreases development (Alliston et al., 1965; Elliot & Ulberg, 1971). In cattle, 2-cell embryos were more susceptible to effects of heat shock than morulae (Ealy et al., 1995).

Ability of the early embryo to withstand elevations in temperature may be contingent upon an increase in cell number or the developmental acquisition of thermoprotective biochemical mechanisms. In most cell types studied thus far, biochemical mechanisms implicated in thermoprotection of cells include members of the HSP70 family and antioxidants such as the thiol peptide, GSH. HSP70 presumably protects cells from heat shock by refolding damaged proteins and stabilizing ribosomal

RNA (Duncan & Hershey, 1989; Nover & Scharf, 1991) whereas glutathione is thought to limit the effects of free radicals (Loven, 1988). These molecules may also be involved in acquisition of thermal resistance in embryos. Oocytes were more resistant to heat shock when microinjected with HSP70 mRNA (Hendrey & Kola, 1991). Moreover, addition of GSH to culture medium partially reduced inhibitory effects of heat shock on murine (Aréchiga et al., 1994; 1995) and bovine (Ealy et al., 1992) embryos. Inhibition of GSH synthesis blocked induced thermotolerance in mouse embryos (Aréchiga et al., 1995). Cumulus cells may also provide thermoprotection to oocytes because inhibition of protein synthesis caused by heat shock was reduced in oocytes encased with cumulus (Chapter 4). The objective of the present study was to determine whether developmental differences in the magnitude of heat stress induced embryonic mortality are caused by changes in resistance of embryos to elevated temperature. In this regard, responses of oocytes, 2-cell embryos, 4 to 8-cell embryos and compacted morulae to heat shock were compared. An additional goal was to further define the role of cumulus, HSP70 and GSH in thermoprotection of oocytes.

Materials and Methods

Materials

DL-buthionine-[S,R]-sulfoximine (BSO), estradiol, fluorescein diacetate (FDA), heparin, HEPES, hyaluronidase, mineral oil, polyvinyl alcohol, sodium pyruvate and antibiotics were purchased from Sigma Chemical Company (St. Louis, MO). Tissue Culture Medium-199 (TCM-199) was purchased from Specialty Media (Lavallette, NJ). Bovine steer serum and heat treated fetal calf serum (htFCS) were purchased from Pel-Freez (Rogers, AK) and Atlanta Biologicals (Norcross, GA), respectively. Frozen semen

from various bulls was obtained from American Breeders Service (Madison, WI) or prepared locally. The CZB medium was prepared as described by Chatot et al. (1989) modified Tyrode's solutions (HEPES-TL, SP-TL and IVF-TL) were purchased from Specialty Media and prepared as described by Parrish et al. (1986). The CR1aa medium was prepared as described by Rosenkrans et al. (1993). Sterile saline [0.9% (wt/vol) NaCl] was supplemented with 100,000 IU/L penicillin-G and 100 mg/L streptomycin (Pen/Strep). Pituitary-derived follicle stimulating hormone (FSH-P) was purchased from Schering (Kenilworth, NJ). Materials purchased from Fisher Scientific (Fair Lawn, NJ) included acrylamide solution (40%), ethidium bromide (EtBr), sodium salicylate and trichloroacetic acid. X-Ray film was from Fuji (Tokyo, Japan) or Kodak (Rochester, NY). Radiolabeled L-[^{35}S]methionine and L-[^{35}S]cysteine (70%:15% mixture, respectively; specific activity > 1000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). Ampholines were purchased from Pharmacia (Uppsala, Sweden).

In Vitro Maturation, Fertilization and Culture of Embryos

Procedures used to produce in vitro matured, fertilized and cultured embryos were modifications of procedures described previously (Appendix A). Cumulus oocyte complexes (COCs) were matured for 22 h in maturation medium [OMM; TCM-199 supplemented with 10% (v/v) steer serum, 50 μg/ml gentamicin, 0.2 mM sodium pyruvate, 2 μg/ml estradiol and 20 μg/ml FSH-P; 10 COCs/50 μl] and then fertilized as previously described. Eight to 10 h after addition of sperm, putative zygotes were vortexed and washed extensively in HEPES-TALP to remove cumulus cells and associated spermatozoa. Putative zygotes were placed in CR1aa (10 zygotes/50 μl) covered with mineral oil and allowed to develop further. On d 5 post-IVF, microdrops of

CR1aa were supplemented with 5 µl of neat htFCS. Cleavage and development to the blastocyst stage were recorded on d 3 and d 7-9 post-IVF, respectively.

Developmental Changes in Embryonic Responses to Heat Shock

Within a given replicate, 2-cell embryos, 4 to 8-cell embryos [28-29 hours post-insemination (hpi)] and compacted morulae (d 5) were washed once in HEPES-TALP and cultured in CR1aa (up to 10 embryos/50 µl) at 39 or 41°C for 12 h. Following heat shock, embryos were cultured continuously at 39°C. Development to the blastocyst stage was recorded on d 7-9 post-IVF. The experiment was replicated using a total of 3-7 microdrops/treatment on 3-5 occasions (n=34-69/treatment).

Differential Effects of Heat Shock on Oocytes and 2-Cell Embryos

Cumulus oocyte complexes were placed in OMM and cultured at 39 or 41°C for the first 12 h of maturation. Subsequently, all further steps of maturation (12-22 h post collection), fertilization and embryonic development were performed at 39°C. Two-cell embryos produced from COCs maintained at 39°C were collected at 28-29 hpi and cultured at 39 or 41°C for 12 h followed by 39°C. Development to blastocyst stage was recorded. The experiment was replicated using 3-6 microdrops/treatment on 3 occasions (n=124-135 oocytes and n=90-100 2-cell embryos/treatment).

To determine effects of heat shock on subsequent development of 2-cell embryos, embryos were collected at 28-29 hpi and cultured at 39 or 41°C for 12 h. At 24 h following the end of heat shock, the number of embryos $\geq$ 4-cell stage was recorded. The experiment was replicated in 3-7/microdrops/treatment on 5 occasions using a total of 51 embryos.

Membrane Integrity of Oocytes, 2-Cell, 4 to 8-Cell Embryos Following Heat Shock

Oocytes or 2-cell and 4 to 8-cell embryos obtained 28-29 hpi were cultured at 39 or 41°C for 12 h followed by 39°C. At 12 h following end of heat shock, membrane integrity was assessed using FDA/ethidium bromide to distinguish membrane-intact cells from membrane-permeable cells (Edwards & Hansen, 1996). The experiment was performed one time using 37 and 49 oocytes, 5 and 9 2-cell embryos and 15 and 16 4- to 8-cell embryos at 39 and 41°C, respectively.

Effects of Heat Shock and Depletion of Glutathione during Oocyte Maturation

Treatments were arranged in a 2 x 2 factorial with main effects of temperature (39 versus 41°C) and BSO (+ or -). Freshly collected COCs were placed in maturation medium (10/microdrop) containing ± 1 mM BSO. Control oocytes were cultured continuously at 39°C. Heat shocked oocytes were cultured at 41°C for the first 12 h of maturation and then cultured for the remaining 10 h at 39°C. Following maturation, fertilization and culture of embryos, number of cleaved embryos and blastocysts was recorded on d 3 and d 7-9, respectively. This experiment was replicated using 6-9 microdrops/treatment on two occasions (n=138-159/treatment).

Total Intracellular Protein and HSP70 Synthesis in Heat Shocked Oocytes

Immediately following collection, oocytes were either denuded by vortexing for 7 min in 50 µl HEPES-TALP containing 300 µg/ml hyaluronidase or left with intact cumulus (COCs). COCs and oocytes were then transferred to 50 µl of CZB medium covered with mineral oil and containing 10% FCS and 50 µCi [^{35}S]methionine and [^{35}S]cysteine (20 oocytes/drop). Oocytes were cultured at 39 or 41°C for 12 h. Following heat shock, COCs were denuded and checked microscopically to ensure

removal of cumulus cells. Both groups of oocytes were washed separately three times in CZB + 0.1% (w/v) polyvinyl alcohol and transferred in a minimum volume to 50 µl of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol and frozen at -70°C until analysis.

Incorporation of radiolabel into intracellular proteins was determined by TCA precipitation (Mans & Novelli, 1961). Proteins from solubilized oocytes were analyzed using 2-D SDS-PAGE as previously described by Edwards and Hansen (1996). Each gel was loaded with 61,000-77,000 dpm of TCA-precipitable radioactivity and exposed to x-ray film; within each replicate (n=4), equal amounts of radioactivity were loaded for control and heat shock treatments and films were exposed for the same amount of time. Quantitative estimates of radioactivity were obtained using densitometric analysis (Chapter 4). Intensity of spots corresponding to HSP68, P71 and P70 were expressed on a per oocyte basis.

Statistical Analysis

Data were analyzed by least squares analysis of variance using the General Linear Models procedure of SAS (1989) and are presented as least squares means ± SEM. Each experiment was performed on several different days, using one or more microdrops (replicates) of embryos or oocytes per treatment on each day. All effects were considered fixed. To avoid statistical problems associated with percentage data, numbers of embryos cleaved or developed to the blastocyst stage within a microdrop were used as the dependent variable in statistical models and total number of embryos or oocytes per drop were used as a covariate to adjust for differences in initial number. To ensure ease of comparison to other developmental data in the literature, data were also analyzed as

percentage cleaved or percentage developed to blastocyst (calculated within a microdrop) and are presented this way in the paper. Both analyses gave very similar probability estimates; estimates reported in the paper are based on analysis of number developing. TCA-precipitable radioactivity was estimated on a pool of oocytes derived from one microdrop; data are expressed on a per oocyte basis. Similarly, the peak volume obtained from densitometric analysis for HSP68, P71 and P70 represents analysis of a pool of oocytes; data were log transformed before statistical analysis. Statistical models included sources of variation due to stage of development or BSO, temperature and replicate.

Results

Developmental Changes in Embryonic Responses to Heat Shock

Exposure to 41°C for 12 h greatly reduced the number of 2-cell ($P<0.005$) and 4 to 8-cell ($P<0.02$) embryos that developed to the blastocyst stage (Figure 6-1). In contrast, subsequent development of compacted morulae was unaltered by heat shock.

Differential Effects of Heat Shock on Oocytes and 2-Cell Embryos

As shown in Figure 6-2, deleterious effects of heat shock were not evident when oocytes were exposed to 41°C during the first 12 h of maturation; however, heat shock greatly reduced the number of 2-cell embryos developing to the blastocyst stage (stage x temperature $P<0.002$). A second experiment was conducted to define the developmental period when heat shock reduces development of 2-cell embryos. Exposure of 2-cell embryos to 41°C for 12 h reduced the number of embryos that were at the 4-cell stage or greater 24 h after the end of heat shock (88 versus 62%; SEM=12; $P<0.01$).

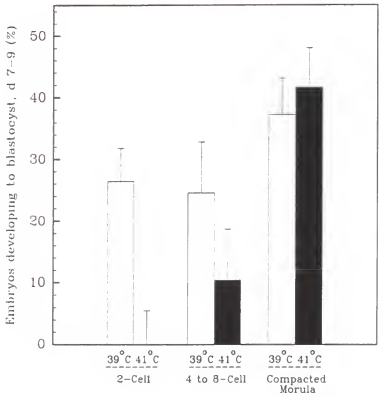

Figure 6-1. Percentages of embryos developing to blastocyst following exposure of two-cell embryos, 4 to 8-cell embryos and compacted morulae to 41°C for 12 h. The total number of embryos per treatment varied from 34-69. Exposure of embryos to heat shock reduced the number of 2-cell and 4 to 8-cell embryos developing to blastocyst but did not alter the number of morulae that developed (stage x temperature interaction: $P<0.05$ for 2-cell versus compacted morula and $P<0.1$ for 2-cell and 4 to 8-cell embryos versus compacted morulae).

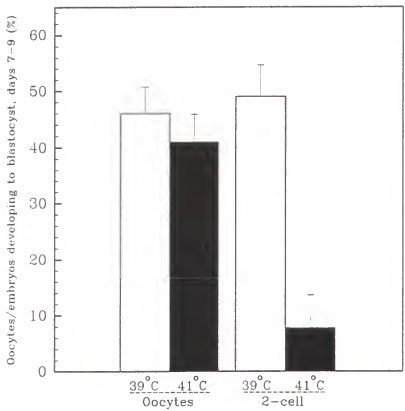

Figure 6-2. Percentages of oocytes and 2-cell embryos developing to blastocyst following exposure to 41°C for 12 h. The total number of oocytes per group ranged from 124-135 and 2-cell embryos per group from 90-100. Heat shock decreased the number of 2-cell embryos developing to the blastocyst stage but did not alter the development of oocytes (stage x temperature $P<0.002$).

Membrane Integrity Embryos Following Heat Shock

Heat shock for 12 h did not alter membrane integrity of oocytes, 2-cell embryos or 4 to 8-cell embryos when determined 24 h following heat shock. All oocytes and embryos examined had intact membranes regardless of treatment.

Effects of Heat Shock and Depletion of Glutathione during Oocyte Maturation

Exposure of COCs to BSO for 24 h or to 41°C for the first 12 h of maturation did not alter the number of cleaved embryos assessed on d 2 post-IVF (Figure 6-3, top panel). Both BSO and exposure to 41°C decreased the number of oocytes developing to the blastocyst stage by d 7-9 following fertilization (Figure 6-3, bottom panel). The decrease in development caused by heat shock was less for BSO because development was already low (temperature x BSO, $P<0.05$).

Total Protein and HSP70 Synthesis in Heat Shocked Oocytes

The total amount of intracellular radiolabeled proteins synthesized by the oocyte was affected by temperature ($P<0.09$) and association of intact-cumulus cells with the oocyte ($P<0.0001$; Figure 6-4). Exposure of denuded oocytes to 41°C for 12 h reduced the synthesis of incorporation of radiolabel into intracellular proteins. In contrast, when cumulus remained encased around the oocyte, heat shock did not alter the total amount of incorporation of radiolabel into intracellular proteins synthesized by the oocyte.

Representative profiles of intracellular proteins synthesized de novo by oocytes at 39 and 41°C are presented in Figure 6-4. Qualitatively, radiolabeled protein synthetic patterns did not differ for oocytes cultured with or without cumulus. Three proteins believed to belong to the HSP70 family were identified based on their similarity to HSP70 proteins previously described in bovine oocytes and 2-cell embryos (Chapter 4)

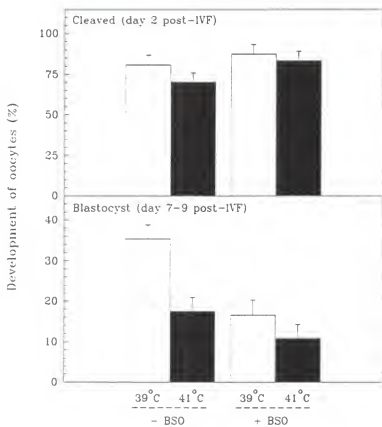

Figure 6-3. Effects of BSO and heat shock during oocyte maturation on subsequent development. The total number of oocytes cultured per group ranged from 138 to 159. Neither heat shock nor BSO altered the number of oocytes that had cleaved by d 2 post-IVF (top panel). Culture of oocytes with BSO or at 41°C decreased the number of oocytes developing to the blastocyst stage by d 7-9 following fertilization (temperature x BSO, $P<0.05$; bottom panel).

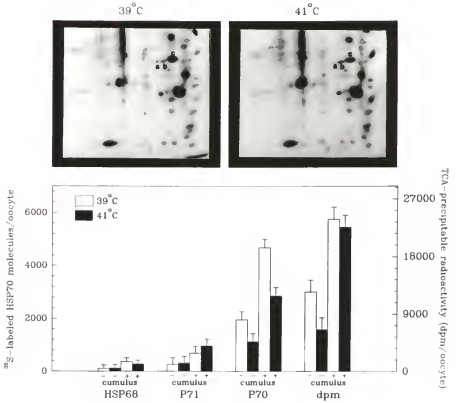

Figure 6-4. Effects of heat shock and removal of cumulus on patterns of intracellular proteins synthesized by oocytes. The top panels are representative fluorographs showing [^{35}S]-labeled proteins produced by oocytes cultured at either 39 or 41°C for 12 h. The particular example is from oocytes cultured with cumulus intact and then denuded prior to analysis by TCA-precipitation and 2-D SDS-PAGE. The pattern of proteins was qualitatively similar for oocytes cultured without cumulus. Synthesis of HSP68, P71 and P70 are labeled a, b and c, respectively. The bottom graph shows results of densitometric analysis of HSP68, P71, P70 and the total amount of TCA-precipitable radioactivity synthesized during culture by oocytes cultured with cumulus-intact (+) or denuded (-) prior to treatment. Results are least squares means $\pm$ SEM of four replicates. Heat shock did not alter the synthesis of HSP68 or P71 but decreased amounts of newly synthesized P70 and the total amounts of intracellular proteins that were synthesized by oocytes ($P<0.09$). Presence of cumulus cells increased synthesis of P71 and P72 ($P<0.06$ and $P<0.0001$, respectively) and the total amount of proteins synthesized ($P<0.0001$).

These proteins were HSP68, whose synthesis in 2-cell embryos is increased by heat shock, and P71 and P70, whose pI and M_r are indicative that they are HSP70. Radiolabeled HSP68, P71 and P70 were detectable in all oocytes regardless of treatment (Figure 6-4). Results of densitometric analysis indicated that heat shock did not alter the synthesis of HSP68 or P71 but decreased synthesis of P70 ($P<0.001$). De novo synthesis of P71 and P70 was dependent on the presence of intact cumulus cells associated with the oocyte because denuding before radiolabeling reduced amounts of P71 ($P<0.06$) and P70 ($P<0.0001$). Amounts of HSP68 also tended to be reduced by denuding but this effect was not significant.

Discussion

These results indicate that the oocyte and early embryo undergo a biphasic developmental pattern of resistance to heat shock, with fertilization and cleavage to the 2-cell stage being associated with increased thermal sensitivity. This is followed by restoration of resistance to elevated temperatures by the morula stage of development. These results imply that infertility caused by heat stress is due at least in part, to effects of elevated temperatures on the embryo and that the decline in deleterious effects of maternal heat stress as pregnancy proceeds (Dutt, 1963; Tompkins et al., 1967; Ealy et al., 1993) is related to differences in the thermal sensitivity of the embryo.

Responses of oocytes exposed to 41°C varied according to the experiment being conducted. Data presented in Chapter 4 demonstrated that exposure of oocytes to 41°C for 12 h reduced subsequent development of oocytes to blastocyst (30 vs 10%). Similar findings were noted in one experiment of the present study (35 vs 18%; Figure 6-3). However, there was no effect of elevated temperature in the experiment in which

responses of COCs and 2-cell embryos to heat shock were compared (46 vs 41% in oocytes and 49 vs 8% in 2-cell embryos). Disparity of results may reflect variation in the quality of oocytes obtained from the abatoir or subtle differences in culture technique or conditions. In any case, the 2-cell embryo is more sensitive to heat shock than the oocyte because when ascertained across all studies, reduction in development caused by heat shock of oocytes generally was of a lower magnitude than for the 2-cell embryo. In vivo, too, the oocyte may be resistant to heat stress; Woody and Ulberg (1967) found that pregnancy rates following insemination were not different between ewes receiving oocytes from heat-stressed donors as compared to those receiving oocytes from nonstressed donors.

The effects of heat shock on the 2-cell embryo occur very early in development because heat shock reduced the number of embryos that reached the 4-cell stage. Similar findings have been reported in the mouse (Elliott & Ulberg, 1971). The loss of thermal resistance associated with fertilization and cleavage is likely the result of biochemical changes in the embryo that lead to the depletion of intracellular concentrations of themoprotective molecules. Indeed, HSP70 and GSH have been implicated in cellular resistance to heat shock (Mitchell et al., 1983; Loven, 1988; Riabowol et al., 1988; Hendrey & Kola, 1991; Nover & Scharf, 1991; Aréchiga et al., 1995) and are present in higher amounts in the mouse oocyte when compared to the 2-cell embryo (Manejwala et al., 1991; Gardiner & Reed, 1994). Thus, even though oocytes can not synthesize increased amounts of HSP68 in response to heat shock (Chapter 4), they may have higher basal amounts than 2-cell embryos because of prestored mRNA, protein, or GSH.

Cumulus cells may also provide protection to the oocyte from elevated temperatures and, if so, loss of cumulus following ferilization and cleavage may increase thermal sensitivity of 2-cell embryos. Present data are consistent with previous evidence that association of cumulus cells with the oocyte during maturation enhances the protein synthetic capability of the oocyte (Chian & Sirard, 1995; Chapter 4). Also, removal of the cumulus decreased synthesis of HSP68, P71 and P70 and this may make the oocyte more susceptible to heat stress. It is also possible that cumulus cells transfer GSH or amino acid precursors of GSH to the oocyte through gap junctions. Caution must be made in interpreting results of cumulus removal experiments because of the possibility of mechanical damage to the oocyte.

There was some evidence that embryos begin to regain thermoresistance as early as the 4 to 8-cell stage of development because there was a nonsignificant tendency for heat shock to compromise development of these embryos less than for 2-cell embryos (26 vs 0% for 2-cell and 25 vs 10% for 4 to 8-cell embryos). Certainly by the morula stage, embryos have acquired increased thermal resistance. Similar findings have been reported by Ealy et al. (1995). There are at least two possibilities for why embryos become more thermotolerant as they proceed in development. One possibility is increased cell number. If the effect of heat shock is to alter the function of 50% of the blastomeres, 2-cell embryos would be left with only one blastomere to form a viable embryo whereas a morula or blastocyst would have 30-50+ viable blastomeres to continue in development. Increased cell number is also associated with increased survival after loss of blastomeres following embryo splitting (Williams et al., 1984). A second possibility is that embryos acquire biochemical mechanisms for thermoprotection during development. Bovine

embryos can increase synthesis of HSP68 in response to heat shock as early as the 2-cell stage (Chapter 4). However, there may be developmental differences in the magnitude of heat-induced HSP68 synthesis or in the amplitude of other heat shock proteins. Early cleavage stage mouse embryos have a limited capacity to synthesize GSH when compared to blastocysts (Gardiner & Reed, 1995) and thermoprotective actions of GSH have been well documented in many cell types (Mitchell et al., 1983; Loven, 1988) including early embryos (Ealy et al., 1992; Aréchiga et al., 1994; 1995).

The role of GSH in oocyte resistance to heat shock was evaluated by testing effects of BSO, a specific inhibitor of GSH synthesis (Griffith & Meister, 1979), on oocyte resistance to heat shock. A clear role for GSH in oocyte or early embryonic function was demonstrated because BSO reduced the number of oocytes capable of forming blastocysts. Additional support for this idea comes from an experiment in which cysteamine, which increases intracellular GSH (Matos et al., 1995), increased the number of oocytes developing to the blastocyst stage following fertilization. Similar effects of BSO as found in the present study for oocytes have been reported for six to eight-cell bovine embryos (Takahashi et al., 1993). Glutathione is probably important for the oocyte because of its roles in scavenging free radicals (Loven, 1988), sperm nuclear decondensation (Calvin et al., 1986) and pronuclear formation (Yoshida et al., 1993). While GSH was clearly important for the oocyte, the general reduction in oocyte developmental competence caused by BSO made it less clear whether depletion of GSH made oocytes more sensitive to heat shock. Perhaps, treatment with BSO did not increase the severity of heat shock because overall effects of BSO at both 39 and 41 °C were severe.

In conclusion, there is a biphasic change in resistance to elevation in temperature as oocytes mature, become fertilized and proceed through development. Resistance declines from the oocyte to the 2-cell embryo and then increases. Evidence suggests a role for cumulus cells in providing protection from heat shock; this effect may be mediated by allowing for increased synthesis of HSP70 and other proteins. Additionally, results support a role for GSH in oocyte function Mechanisms responsible for increased resistance as 2-cell embryos proceed to the morula stage are still undefined. The fact that heat-inducibility of HSP68 occurs as early as the 2-cell stage indicates that other factors in addition to HSP70 may be involved.

CHAPTER 7
GENERAL DISCUSSION

Early embryonic development proceeds under the direction of maternal mRNA formed during oogenesis. Transcription of cultured bovine embryos is not initiated until the late 4-cell or 8-cell stage of development (Camous et al., 1986; King et al., 1988; Barnes & First, 1991). This finding implied that the early embryo would have a limited ability to respond to changes in its environment because transcriptional control is absent. Acquisition of embryonic thermotolerance was associated with ability to undergo transcription and synthesize thermoprotective molecules such as HSP70. This hypothesis was raised at the end of Chapter 2 and is graphically illustrated on the top panel of Figure 7-1. However, data described in this thesis demonstrate that environmental signals can activate certain genes before EGA is commonly thought to occur and therefore the working model to understand acquisition of embryonic thermotolerance needs to be modified to reflect this fact (Figure 7-1; bottom panel). Central to this reappraisal of the working thesis was the finding that bovine embryos can respond to heat shock by modifying the synthesis of a specific protein, HSP68, as early as the 2-cell stage (Chapters 4 and 5). This observation was remarkable because it suggests that heat shock induces alterations in translation, mRNA stability or transcription of the early embryo.

Data from other systems also support the idea that early embryos can respond to changes in their microenvironment by alterations in protein synthesis. Transcriptional activity of early mouse embryos is affected by their manipulation and culture (Vernet et

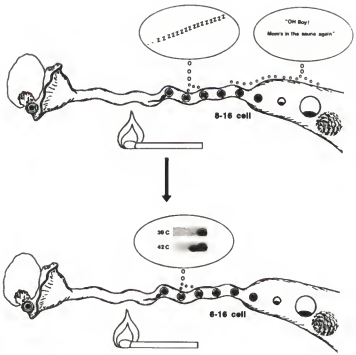

Figure 7-1. Model depicting responses of bovine embryos to heat shock. The premise that sensitivity of embryos prior to the 8 to 16-cell stage to elevated temperature (represented by lit match) is a result of an inability to respond to environmental changes (represented by ..zzzzzzzz..) is not correct (top panel). Rather, bovine embryos can respond to elevated temperature by modifying the synthesis of HSP68 as early as the 2-cell stage.

al., 1993). Moreover, transient expression of HSP70.1 during EGA was much higher in embryos that developed in culture when compared to those developed in vivo (Christians et al., 1995). Thus, the idea of a static embryo unable to respond to environmental changes before EGA is not appropriate.

Stage specific differences in the regulation of heat-induced synthesis of HSP68 existed (Chapter 5). By the 4-cell stage, heat-induced synthesis of HSP68 is clearly the result of alterations in transcriptional activity because α-amanitin blocked heat shock response. In contrast, HSP68 synthesis in 2-cell embryos which was α-amanitin resistant, may in large part be due to alterations in translation or mRNA stability. Of these possibilities, increased mRNA stability is least likely. Maternal pools of mRNA decrease dramatically following resumption of meiosis and are at very low levels by the 2-cell stage (Paynton et al., 1988). In fact, most hsp70 mRNA is depleted before the 2-cell stage in the mouse (Manejwala et al., 1991). Heat-induced increases in the translational efficiency of hsp70 mRNA may be the most likely explanation because this phenomenon has been reported for other heat shock proteins (Colbert & Young, 1987; Nover & Scharf, 1991). Transcriptional activation increasing HSP70 in 2-cell embryos remains a possibility, however. Expression of hsp70 is regulated at the level of elongation rather than through binding of RNA polymerase II to the promoter in *Drosophila* (Gilmour & Lis, 1986). If such a control mechanism was operational in 2-cell embryos, it would be insensitive to inhibition by α-amanitin because this inhibitor acts to block transcription by preventing RNA polymerase II from binding the promoter region of the gene (Wieland & Faulstich, 1978).

The fact that both 2-cell and 4-cell embryos were collected at 28-29 hpi establishes that stage specific differences in regulation of heat-induced synthesis of HSP68 are related to cleavage rather than time following insemination. Though not proven, transcriptional dependent synthesis of HSP68 in 4-cell embryos could be related to mechanisms implicated in EGA (Chapter 2; Section V). Possibilities include alterations in chromatin structure that might allow for access of transcriptional factors and RNA polymerase II to the promoter region of the HSP68 gene, dilution of cytoplasmic factors, alterations in cell cycle components, induction of post-translational modifications of proteins through kinase-mediated phosphorylation, and changes in acetylation status of histones.

The oocyte is susceptible to the deleterious effects of heat shock; effects on subsequent development following fertilization are most prominent when occurring during the first 12 h of maturation and include an inhibition of protein synthesis (Chapter 4). However, when compared within the same experiment, effects of heat shock on subsequent development of oocytes to the blastocyst stage following fertilization were minimal compared to effects on 2-cell embryos (Chapter 6), suggesting a loss of thermoprotective mechanisms following fertilization and cleavage.

Intimate association of cumulus cells with the oocyte partially ameliorated deleterious effects of heat shock (Chapters 4 and 6) implying a thermoprotective role of cumulus cells during maturation (Figure 7-2). Exposure of oocytes to heat shock reduced synthesis of intracellular proteins; percent reduction was greater if cumulus were removed prior to treatment. The requirement of intimate association of the oocyte with its companion cumulus cells has been well described (Buccione et al., 1990; Eppig,

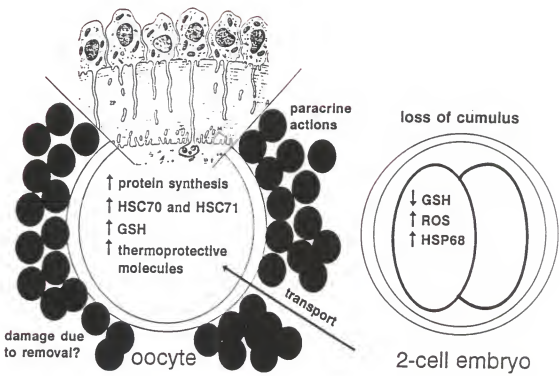

Figure 7-2. Model illustrating possible thermoprotective actions of cumulus cells during oocyte maturation. The oocyte is intimately associated with it companion cumulus cells. Removal of cumulus decreased overall protein synthesis and synthesis of HSP70; thus, cumulus may protect oocytes from heat shock in part by promoting overall protein synthesis and synthesis of specific heat shock proteins. Cumulus cells may also act to secrete paracrine factors that could aid in the transport of substrates via gap junctions to the oocyte and allow for increased synthesis of thermoprotective molecules such as HSP70 and GSH. Loss of cumulus cells prior to or following fertilization and cleavage could be associated with increased sensitivity of the 2-cell embryo by allowing increased production of reactive oxygen species (ROS) and decreased GSH content.

1994). There are several possible mechanisms through which cumulus could enhance thermostability of oocytes. Culture of oocytes with cumulus cells intact increased overall protein synthesis and amounts of HSC71 and HSC70 compared to those denuded prior to treatment (Chapter 6). Cumulus mediated effects may also involve paracrine factors that aid in the transport of substrates via gap junctions and allow for increased synthesis of thermoprotective molecules within the oocyte such as HSP70 and GSH. Caution must be exercised when interpreting data on effects of cumulus. Denuding itself may damage the oocyte. Regardless, if cumulus are involved in thermoprotection of the oocyte, their loss prior to or following fertilization and cleavage of the early embryo may be associated with increased thermal sensitivity of the 2-cell embryo.

The oocyte and early embryo undergo a biphasic developmental pattern of resistance to heat shock (Chapter 6). Differences in thermal sensitivity of embryos are noted as early as the second cleavage division. This is followed by restoration of resistance to elevated temperatures by the morula stage of development. Other evidence to support the idea of developmental acquisition of thermotolerance is provided by examining the effects of heat shock on protein synthesis (Chapter 4) and evaluating the severity of heat shock required to elicit a heat shock response at the blastocyst stage in mice (Chapter 3). With the exception of the 8-cell embryo, exposure to heat shock reduced the total amount of intracellular proteins synthesized by embryos until the expanded blastocyst stage; thereafter, heat shock increased protein synthesis. Moreover, exposure of mouse blastocysts to 40°C was insufficient to induce synthesis of heat shock proteins compared to 8-cell embryos. Cumulatively, these results are in agreement with Ealy et al. (1995) and imply that infertility caused by heat stress may in part be due to

effects of elevated temperatures on the early embryo and that the decline in deleterious effects of maternal heat stress as pregnancy proceeds is related to differences in the thermal sensitivity of the embryo.

There are at least two possibilities for why embryos gain resistance to elevated temperature as they proceed in development. One possibility is increased cell number. If the effect of heat shock is to alter the function of 50% of blastomeres, 2-cell embryos would be left with only one to form a viable embryo whereas morulae or blastocysts would have 30-50+ viable blastomeres to continue in development. A more likely explanation is that embryos acquire biochemical mechanisms for thermoprotection as they proceed in development.

Greater sensitivity of cleavage stage embryos to elevated temperature was not related to an inability to synthesize increased amounts of HSP68 following heat shock. For example, 2-cell embryos which are clearly very sensitive to disruption by heat shock can respond to elevated temperature by synthesizing increased amounts of HSP68 (Chapter 4). Such a response was noted for embryos at all stages of development examined (Chapter 5). In contrast, oocytes synthesized comparable amounts of HSP68 when cultured at 39 and 42°C.

With the exception of matured oocytes, constitutive synthesis of HSC71 and HSC70 was detected in embryos cultured at 39 and 42°C. Heat-inducibility was noted only at the expanded blastocyst stage which is coincidental with period of time during development when heat shock first fails to decrease protein synthesis (Chapter 5). Thus, it is possible that changes in regulation of constitutive synthesis of HSP70 is associated with increased thermotolerance. Also, one can not rule out the possibility that stage-

specific differences exist between embryos in the amount of HSP70 that is produced. Experimental design of the studies detailed in this thesis did not allow for quantification of HSP70 at different stages of development.

Studies described in this thesis measured de novo synthesis of proteins only and not amounts of prestored protein. Thus, it is possible that oocytes are more resistant to elevated temperature when compared to 2-cell embryos because prestored levels of HSP70 are relatively high and then decrease following resumption of meiosis and fertilization (mouse; Manejwala et al., 1991). Data presented in Chapter 4 indicate that hsp70 mRNA follows a similar pattern in bovine oocytes. Oocytes synthesized HSP68 at 39 and 42°C; this protein was either absent or present in very low amounts in 2-cell embryos cultured at 39°C. Synthesis of HSP68 in oocytes cultured at 39°C may represent normal translation of prestored hsp70 mRNA or alterations in mRNA stability or increased translation following stress of denudement of cumulus.

Evidence presented in Chapter 3 using mouse embryos also points out that factors in addition to HSP70 are required for thermotolerance. For example, murine embryos can undergo induced thermotolerance at the 8-cell stage if development occurs in culture but not until the blastocyst stage if development occurs in utero (Ealy & Hansen, 1994). Fetal calf serum is required for induced thermotolerance. These stage-specific and serum-related differences are not related to qualitative differences in heat-induced synthesis of HSP70. Synthesis of HSP70 was not dependent upon addition of FCS to culture medium and 8-cell embryos synthesized HSP70 following a heat shock of 40°C regardless of whether they developed in utero or in culture (Chapter 3). Similarly, Kapron-Bras and Hales (1992) showed that variation in development of cross-tolerance

in postimplantation mouse embryos from two different strains (BALB/c and SWV) was not correlated with differences in heat-induced synthesis of HSP68. In fact, heat-induced increases in HSP68 were less in the thermotolerant strain.

Given that no qualitative differences in the amounts of heat-inducible HSP68 were detected between bovine and mouse embryos at various stages raises the question as to why later stage embryos are more tolerant of elevated temperatures than earlier ones. One possibility is that developmental changes in GSH synthesis are involved. Following resumption of meiosis, GSH content in the mouse embryo decreases continuously to the blastocyst stage (7 mM to 0.7 mM; Gardiner & Reed, 1994; See Figure 7-2). The precipitous decline in GSH is coincident with increased thermal sensitivity of the 2-cell embryo. Ability to synthesize increased amounts of GSH is not noted until the blastocyst stage (Gardiner & Reed, 1995). Moreover, supplementation of GSH (Ealy et al., 1992; Aréchiga et al., 1995) or an inducer of GSH synthesis (Aréchiga et al., 1995) to culture medium increases resistance of bovine and murine embryos to heat shock. The converse is true when mouse morulae are depleted of glutathione during heat shock (Aréchiga et al., 1995).

To conclude, a model depicting events associated with acquisition of thermotolerance in bovine preimplantation embryos is presented in Figure 7-3. Two-cell embryos are more sensitive to elevated temperature than oocytes suggesting a loss of thermoprotective mechanisms associated with fertilization and cleavage. Increased thermal resistance is noted as early as the second cleavage division. Thereafter, as embryos proceed in development, thermal sensitivity decreases. Developmental changes in thermotolerance are not related to changes in heat-induced synthesis of

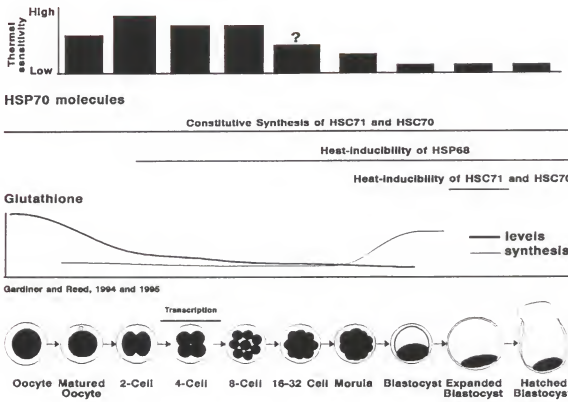

Figure 7-3. Current model of events associated with acquisition of thermotolerance in bovine preimplantation embryos. 2-cell embryos are more sensitive to elevated temperature than oocytes suggesting a loss of thermoprotective mechanisms associated with fertilization and cleavage. As embryos proceed in development, thermal sensitivity decreases. Increased thermal resistance is noted as early as the second cleavage division. Developmental changes in thermotolerance are not related to changes in HSP68 synthesis which begins as early as the 2-cell stage. Constitutive synthesis of HSC71 and HSC70 occurs at all stages of development examined but heat-inducibility of these proteins only occurs at the expanded blastocyst stage. Thus, it is possible that changes in the regulation of constitutive HSP70 is associated with increased thermotolerance. A second possibility is that acquisition of thermotolerance is related to GSH. Based on published data using the mouse, following resumption of meiosis, GSH content continuously declines and is approximately 10-fold lower by the blastocyst stage of development. Note the precipitous decline in GSH content is coincident with increased thermosensitivity of the 2-cell embryo. Embryos do not acquire the ability to synthesize GSH until the blastocyst stage.

HSP68 which begins as early as the 2-cell stage. Constitutive synthesis of HSC71 and HSC70 occurs at all stages of development examined but heat-inducibility of these proteins only occurs at the expanded blastocyst stage. Thus, it is possible that changes in the regulation of constitutive HSP70 is associated with increased thermotolerance. A second possibility is that acquisition of thermotolerance is related to GSH. Based on published data using the mouse (Gardiner & Reed, 1994; 1995), following resumption of meiosis, GSH content continuously declines and is approximately 10-fold lower by the blastocyst stage of development. Note the precipitous decline in GSH content is coincident with increased thermosensitivity of the 2-cell embryo. Embryos do not acquire the ability to synthesize GSH until the blastocyst stage.

A third possibility is that acquisition of thermotolerance is related to other yet unidentified molecules. Identification of such molecules and further characterization of GSH and HSP70 during developmental acquisition of thermotolerance could result in novel approaches for protecting embryos from deleterious effects of maternal hyperthermia.

APPENDIX A
BOVINE IN VITRO/MATURATION/FERTILIZATION/CULTURE PROCEDURES

I. Procedures

These procedures are modifications of existing procedures of Parrish et al. (1986), Xu et al. (1992), and Hernandez-Ledezema et al. (1993).

Helpful Hints for Successful IVF:

1. All media should be prewarmed to 39°C before use in the following manner:

   a) If media are prepared such that they are to be used in the culture room, outside of the incubator, prewarm for at least 2 to 3 h in oven set at 39°C. Make sure lid is on tight. Placement of these media in a 5% CO_2 environment may result in an alteration of pH that could be detrimental to embryonic viability. Medium to be prewarmed in this manner includes Saline, Hepes-TALP, Sp-TL, Sp-TALP and Oocyte Collection Medium.

   b) If media are prepared for use in a 5% CO_2 environment as would be any medium used for embryo or cell culture, prewarm to 39°C in an incubator. In order for the pH of the medium to be equilibrated it is important to loosen the lid on any bottle of medium placed in the incubator. Leaving media buffered for a specific CO_2 environment outside the incubator too long will result in a dramatic change of pH which could severely affect embryonic viability and development. Media that must be prewarmed in an incubator to adjust the pH include: OMM, IVF-TALP and CR1aa.

2. Before looking at embryos using the microscopes, make sure stage has been prewarmed. Metal is an excellent conductor of cold from one material to another. Placement of a dish on a cold lab bench or microscope stage could result in a rapid cold shock of embryos. Likewise when washing oocytes, place beakers on plastic mesh as opposed to cold counter top. Whenever possible place dishes containing putative zygotes (O/E) on slide warmer!!!!

3. Due to repeated opening and closing of incubator door, temperature at the front of the incubator fluctuates. Thus placing dishes containing O/E at the back of the incubator reduces exposure to changes of temperatures.

4. Pipette tips may contain toxins or other substances that might inhibit development of embryos. As a precaution always rinse pipette tip at least once before adding new medium to a microdrop containing embryos.

A. Collection of Ovaries and Retrieval of Oocytes

1. Before leaving for the slaughterhouse, place all media to be used for oocyte collection and culture in 39°C oven. Prepare an appropriate number of 50 µl drops of OMM in 60 x 15 mm culture dishes (6-10/dish) and cover with embryo tested mineral oil. Place in incubator at 39°C until needed.

2. Autoclave appropriate amount of saline (~2 L). Once cooled, add 10 mL of Pen/Strep (Stock 25) to one liter of saline (final concentration of Pen/Strep is 1X). Pour approximately 500 mL of saline into each of two sterile ovary buckets. Place the two ovary buckets in a thermos for transport. Add 10 ml Pen/Strep to one liter sterile saline and place in 39°C oven until needed.

3. Take thermos, scissors, hair-net, hard-hat, gloves, boots and lab-coat to slaughterhouse. Remove ovaries from reproductive tract of cows immediately after internal organs are extracted from the carcass and place into one ovary bucket. Collect only ovaries with substantial follicular development.

4. After ovary collection, transfer ovaries from the first container into the second one containing fresh saline with 1X Pen/Strep; place ovary bucket containing ovaries into thermos and transport immediately to the laboratory.

5. Upon return to the lab put on clean gloves and wash ovaries vigorously 4 to 5 times with fresh transport saline prewarmed to 39°C in oven and containing 1X Pen/Strep to remove blood and debris. Following washes, place ovaries in plastic beakers containing fresh prewarmed saline 1X Pen/Strep.

6. Add ~200 mL oocyte collection medium into sterile 400 mL beakers.

7. Put on new gloves and attach a hemostat to the base of the ovary such that the ovary is firmly held in place. Discard fluid of large follicles (> 10 mm) by rupturing and discarding fluid before processing ovaries (follicular fluid promotes clotting of medium). Hold ovary above beaker and make checkerboard incisions (for 2 to 3 mm squares) across entire ovary. To prevent contamination of medium with blood (which promotes formation of clots), do not make checkerboard incisions across corpora lutea.

8. Submerge ovary into oocyte collecting medium and swirl vigorously. Repeat process until a group of 6 to 8 ovaries has been processed in a beaker.

9. Once completed, place beaker in H_2O bath for 4 to 5 min to allow oocytes and debris to settle. Using a 25 mL pasteur pipette, slowly remove all but the bottom 50

mL of medium. Add ~200 mL of fresh oocyte collection medium and repeat process (3 or 4 times) until medium is clear. Remove all but the bottom 50 mL of medium, then transfer into a prewarmed gridded culture dish (100 x 15 mm; Falcon 1012) and place on plate warmer until ready for searching.

Note: To avoid exposure of oocytes to cold shock, when washing oocytes after collection do not place beaker on cold counter; instead, place beaker on plastic mesh. This will avoid rapid conduction of cool temperature of counter to the bottom of the beaker where the oocytes are. Additionally, before searching for oocytes, turn on the microscope ~20 min prior to use so the stage may warm up before placing dish containing oocytes on it. Also, place heater in close proximity to the microscope such that the air is warmer where one is to be working directly with the oocytes.

10. Collect cumulus oocyte complexes (COCs; use of a wiretrol pipette minimizes the amount of debris transferred with the oocytes), as fast as possible to prevent adverse effects of cold shock. Only COCs which have at least one layer of compact cumulus cells and an evenly granulated cytoplasm with no dark spots or clear spaces should be used for subsequent steps. Place COCs retrieved into one 60 x 15 mm culture dish containing oocyte collection medium or Hepes-TALP on the plate warmer.

11. After oocyte retrieval is completed for one dish, wash oocytes an additional time in oocyte collection medium or Hepes-TALP then transfer immediately to microdrops of OMM (10 COCs/drop) that has been prewarmed at 39°C in incubator for at least 2-3 h. Repeat process for additional plates until all oocytes are collected. It is essential that oocytes be collected, washed and placed in OMM as quickly as possible.

12. Incubate COCs for 22 h at 39°C.

B. In Vitro Fertilization

1. Prepare appropriate number of 4-well plates (Nunc, #176740) containing 600 µl IVF-TALP/well and incubate at 39°C in incubator at least 2-3 h prior to use.

2. Immediately prior to use, place Hepes-TALP that has been prewarmed to 39°C in oven for at least 2 h into several culture dishes (60 x 15 mm; X-bottomed) and place on plate warmer.

3. Remove one dish containing matured oocytes, retrieve COCs and place in Hepes-TALP. Wash 1 time in Hepes-TALP.

4. Following wash, place ~30 oocytes per well in IVF-TALP (once a 4-well plate is filled immediately return to incubator and get a new one). Repeat process for additional dishes until all oocytes are processed.

5. After all oocytes are in IVF-TALP, remove plates from incubator and add 25 µl sperm suspension (see section I.G) and 25 µl PHE mix (Stock #20). One well should be prepared without sperm, but with PHE, to determine the incidence of parthenogenesis. Incubate for 8-10 h at 39°C.

6. Use the DAPI staining procedure in section II:O to determine rate of oocyte maturation and/or fertilization.

C. IVF: Sperm Preparation

Note: It is critical that spermatocytes not be exposed to cold shock during the following steps; make sure that all media used during these steps are warmed to 39 °C before use. There are two methods available for the preparation of sperm prior to IVF:

1. Percoll Gradient:

a. Place 3 mL of 90% Percoll in a 15 mL conical tube.

b. Slowly layer 3 mL of 45% Percoll on top of the 90%; incubate gradient at 39°C for at least 1 h in oven with the cap on tight.

c. Thaw 2 straws of semen from different bulls in the cyto-thaw for 45-60 seconds.

d. Slowly add thawed semen to the top of Percoll gradient. Place conical tubes in centrifuge carriers that have been prewarmed to 39°C in oven and centrifuge at 2500 rpm for 10 min using the IEC centrifuge.

e. After centrifugation, collect sperm pellet at the bottom of the Percoll gradient. Place in a 15 mL conical tube containing 10 mL Sp-TALP. Centrifuge at 1000 rpm for 5 min.

f. 1) Discard all of the supernatant. Dilute the sperm pellet using IVF-TALP that had been prewarmed in the incubator for at least 1.5 h to a volume required to yield the appropriate number of 25 µl aliquots of sperm to be added to individual wells of the 4-well plates containing oocytes (usually ~1 mL).

2) Or one could determine exact dilution required to add ~1 x 10^6 in 25 µl. To do so, add 10 µl sperm suspension to 90 µl ddH_2O (kills sperm). Load 10 µl of sample onto a hemocytometer. Count the number of sperm in 5 squares (see Laboratory Methods Book). Multiply sperm number by 500,000 to determine concentration per mL.

g. Add 25 µl sperm preparation and 25 µl PHE mix (Stock 20) to each droplet (except parthenogenesis drops).

h. Quickly determine if sperm are motile by using the inverted microscope and place in incubator at 39°C for 8-10 h.

2. Swim-up:

a. Thaw 6 to 8 straws of frozen semen in the cyto-thaw for 60 seconds. If possible use semen from different bulls.

b. Combine contents of straws in 5 mL Sp-TALP. Place sample into the incubator (39°C) for 5 minutes.

c. Centrifuge semen (1000 rpm; 5 min) and discard all but the bottom 1 mL of supernatant.

d. Prepare 4 to 5 test tubes containing 1 mL Sp-TALP. Add approximately 250 µl of sperm suspension very slowly to the bottom of each tube using a 20 ga. needle and 1 mL syringe. Place tubes in incubator (39°C) for 1 h.

e. At the end of sperm swim-up, aspirate the top 800 µl from each tube and combine samples. Centrifuge (1000 rpm) the combined sample for 5 minutes. Discard all but the bottom 500 µl of supernate.

f. Add 10 µl sperm suspension to 90 µl ddH_2O (kills sperm). Load 10 µl of sample onto a hemocytometer. Count the number of sperm in 5 squares (see laboratory materials and methods book). Multiply sperm number by 500,000 to determine concentration per mL. Adjust final concentration to approximately 25 X 10^6 sperm/mL with IVF-TALP.

g. Add 25 µl sperm preparation and 25 µl PHE mix (Stock 20) to each droplet (except parthenogenesis drops) and incubate at 39°C for 8-10 h.

D. Post-IVF: Early and Late Embryonic Development

Note: It is essential that transfer or handling of O/E be done as quickly as possible or subsequent development rates will be affected.

8-10 h Post-IVF

1. After microscope and slide warmer have been warmed sufficiently, remove one 4-well plate containing IVF drops from the incubator and place on slide warmer. Cumulus cells from O/E should be removed. Add 40 µl of Hepes-TALP to a 1.5 mL microcentrifuge tube that has been rinsed out. Add up to 100 O/E to microcentrifuge tube and vortex for 4 min. Rinse tube 3-4 times with Hepes-TALP to remove all O/E. Wash O/E a minimum of 3 times with Hepes-TALP. Then use a

wiretrol pipette to transfer to microdrops (10/microdrop) of CR1aa that had been prepared earlier and prewarmed in the incubator for a minimum of 2-3 h.

2. Repeat until all plates have been processed and O/E have been allotted to microdrops.

Day 3 Post-IVF

1) Prewarm stage of inverted microscope by placing heater near the microscope for at least 30 min prior to use.

2) Assess cleavage rate of embryos by determining the number of embryos cleaved and dividing by the number of O/E placed initially in the microdrops. Return to incubator.

Day 5 Post-IVF

1) Add 5 µl of sterile filtered neat htFCS to each microdrop and return to incubator.

Days 7-9 Post-IVF

1) Assess development of embryos to blastocyst stage.

Time Course for Development of Bovine IVF-derived Embryos

Day of Culture	Expected Stage of Development
1	1- and 2-cell (28-29 hpi)
2	4- and 8-cell
3	8- and 16-cell
4	early morula
5	late morula
6	late morula and early blastocyst
7-9	blastocyst; normal, expanded or hatched

E. Alternative Procedures for use in Embryo Culture

Preparation of Oviductal Cells

1. Collect 1 to 2 oviducts from reproductive tracts and transport in saline with ovaries to the laboratory.

2. Upon return to the lab, put on gloves. Use sterile or clean mincing scissors by submerging in 70% EtOH and cut off all tissues from oviducts except the tip of the uterine horn. Cut off the fibrillar portion of oviducts. Using a gauze pad saturated with 70% EtOH wipe off oviduct.

3. Squeeze oviducts from the isthmus end (smallest end; uterotubular junction) with small sterile forceps into a 60 x 15 mm culture dish containing oviduct culture medium (OCM; < 8 mL).

4. Aspirate oviduct cells once using a 5 mL syringe and 20 ga. needle. Using a sterile plastic transfer pipette, transfer OCM containing oviductal cells to a 15 mL conical tube. Place upright under hood until the bulk of oviductal cells settle (~2-3 min).

5. Using a sterile plastic transfer pipette, discard supernatant containing small cells and debris. To wash oviductal cells, add ~14 mL of OCM that has been prewarmed in the incubator at 39°C with cap loosened to remaining cells in the 15 mL conical tube. Invert conical tube 3 or 4 times. Allow oviductal cells to settle as described previously. Wash oviductal cells a minimum of 4 times using OCM.

6. After final wash, discard all but 1 mL OCM containing oviductal cell pellet. Transfer remaining OCM containing oviductal cells into a 100 X 20 mm culture dish containing ~25 mL of OCM. Place in 39°C incubator and culture until needed.

Oviduct cells may be used as a source of cells for co-culture or to condition medium.

Preparation of Oviductal Cells for Co-culture

a. Remove appropriate amount of oviductal cells from OCM and place in a 15 mL conical tube. Allow the bulk of cells to settle. Discard supernatant. Wash cells in LEC (or appropriate medium) as described in the previous section #5, at least 4 times.

b. After washing for the fourth time, discard supernatant and resuspend oviductal cells in an appropriate volume of LEC to obtain ~10-15 oviductal worms/50 µl of medium (i.e., to 500 µl of cells pelleted add ~14 mL of LEC).

c. Invert conical tube containing LEC and appropriate concentration of cells 3 or 4 times and then immediately add 50 µl of mixture to the first two wells of a 4-well plate. Replace cap and invert tube 3 or 4 times then add 50 µl of mixture to the last two wells of the 4-well plate (oviductal cells settle quickly, in order to maintain approximately the same number of worms/50 µl of medium it is important to remix medium containing cells after every two wells). Cover microdrops with prewarmed (39°C) embryo tested mineral oil. Place in incubator at 39°C until needed.

Microdrops containing oviductal cells are to be prepared at least one day prior to use.

Preparation of Oviductal Conditioned Medium

a. To condition medium, follow steps 1 and 2 listed in **Preparation of Oviductal Cells for Co-culture**. Place 10 mL of medium containing the appropriate amount of

oviductal cells to yield 10-15 worms/50 µl of medium in a T-25 flask. Make sure the lid is loose and place flask containing medium with oviductal cells at 39°C for 24 to 48 h. Suggested time is 48 h.

b. 24-48 h later, sterile filter medium by decanting medium containing oviductal cells into a 20 mL sterile syringe and passing it through a .2 µm filter into a 15 mL conical tube. Conditioned medium may be used immediately to prepare microdrops or stored at 4 C for up to 7 days. Additionally, medium demonstrated to promote good development may be frozen and used at a later time.

BRLC Culture

a. Store Buffalo Rat Liver Cells (BRLC; American Type Culture Collection; BRL-3A; TCC CRL 1422) in the liquid nitrogen tank until use.

b. To begin BRLC culture, remove 1 vial of cells from liquid nitrogen and place in warm water (37 to 40 C) for 40 to 60 seconds. Dip vial into 70% EtOH and place contents in a T25 flask (Corning, #25106). Add 10 mL of Hams-F12 medium (containing 5% BSS and 1% Pen/Strep), lay flask on its side, loosen lid and incubate for 24 h at 37 C.

c. After 24 h, examine cells under the reverse phase microscope in the culture room; cells should have attached to the bottom of the flask and be 30 to 70% confluent. Remove medium and replace with 10 mL Hams-F12 containing additives. Medium must then be changed every 48 to 72 h.

> *Note: Percent confluency is a subjective scoring system to determine whether a monolayer of cells have formed. A complete monolayer, or 100% confluency, is defined as the time when a single layer of cells cover the entire bottom of the flask.*

d. Once BRLC have become 100% confluent (6 to 12 days), sub-culture by removing medium and adding 1 to 2 mL trypsin (Stock 23). Incubate cells at 37 C for 5 min for trypsin to detach cells from the flask.

e. Following incubation, cells still remaining attached to the flask must be removed by manually scraping off cells using a cell scraper (Fisher #08-773-2) or plastic pipette. Place cells (still in trypsin) from each flask into 3 to 4 new T25 flasks, add 10 mL of Hams-F12 plus additives and incubate at 37 C as described previously (as if freshly thawed cells).

f. Cells should not be discarded unless they become contaminated or ample quantities of BRLC exist (in culture or frozen). Instead, cells can be continuously grown and sub-cultured for use in IVF procedures or frozen to replenish BRLC stocks.

g. If cells are discarded, remove medium and treat flasks with bleach for 5 minutes (to kill cells).

h. Freeze BRLC by detaching cells with trypsin, centrifuging at 1500 rpm for 15 min and adding 1 mL Hams F12 (plus 5% BSS and pen/strep) containing 5% DMSO for each flask of cells to be frozen. Aliquot cell suspension in 1 mL volumes to cryo-store tubes (Dynatech, #006-010-0101) and store at -70 C overnight. Place cells into a styrofoam container to reduce the quickness of freezing. The following morning, place frozen cells into liquid nitrogen.

Conditioning Medium with BRLC

a. Medium can be conditioned with BRLC when confluency is between 50% to 100%. However, the greater the confluency, the greater the level of medium conditioning.

b. To condition medium (CZB medium or LEC medium); remove Hams-F12 medium from one flask containing BRLC, wash with 10 mL of the desired medium, then place 10 mL of the desired medium into flask and incubate for 24 to 48 h.

c. Following conditioning, remove medium from flask and sterile filter. Medium can be used immediately or stored at 4 C for up to 7 days before use.

d. Following BRLC-conditioning, cells can be re-used either to condition additional medium or for subculture.

II. Preparation of Media

A. General Notes

1. When making a solution, please place two dates on the bottle if appropriate: the date made and the expiration date. Additionally, label all stocks "FOR IVF".

2. Store all media at designated areas in the walk-in cooler (4 C) or the new laboratory freezer (-20 C).

3. Unless otherwise noted, use Sigma culture grade water.

4. In general, prewarm medium for several hours prior to use; if medium is to be used at room temperature outside of the incubator prewarm in oven at 39°C with lid capped tightly, however, if medium is to be used for the purpose of culturing cells or embryos in the incubator prewarm to 39°C in incubator so that the pH may be equilibrated.

5. For stocks, label every tube.

6. Don't sterile filter solutions if you can autoclave. When autoclaving solutions, mark the volume of each container before autoclaving. After autoclaving, bring the volume of solutions back to their original volume with autoclaved water.

7. During filtration, use the most economical filters for the volume being sterilized. If filtering less than 20 mL, use syringe tip filters. For larger volumes, screw-on bottle filters are the most economical. Generally, filters can be used to sterilize several types of media (eg. TALP solutions).

8. All glassware is to be soaked in either Sigma-Clean (glassware used for salt solutions) or PCC-54 (glassware used for protein medium). Recommended by R.M. Robert's Lab.

B. Stock Solutions

Stock 1: NaCl. Dissolve 6.665 g in 50 mL water. Sterile filter and store at 4 C. Label as "Stock 1: NaCl, date made".

Stock 2: KCl. Dissolve 0.588 g in 50 mL water. Sterile filter and store at 4 C. Label as "Stock 2: KCl, date made".

Stock 3: $NaHCO_3$. Dissolve 1.052 g in 50 mL water. Sterile filter and store at 4 C for one week only. Label as "Stock 3: Bicarb, date made, expiration date".

Stock 4: PO_4. Dissolve 0.235 g $NaH_2PO_4+H_2O$ in 50 mL water. Sterile filter and store at 4 C. Label as "Stock 4: PO_4, date made".

Stock 5: Na lactate. Purchase as a 60% syrup. Store indefinitely at 4 C. Label as "Stock 5: Na Lactate, date purchased".

Stock 6: 1 M HEPES. Add 119 g of HEPES to 400 mL water. Adjust pH to 7.0 and bring volume up to 500 mL. Sterile filter and cover container with aluminum foil; store at 4 C indefinitely. Label as "Stock 6: 1 M HEPES, date made".

Stock 7: $CaCl_2$. Dissolve 1.470 g $CaCl_2+2H_2O$ in 50 mL water. Sterile filter and store at 4 C. Label as "Stock 7: $CaCl_2$, date made".

Stock 8: $MgCl_2$. Dissolve 1.017 g of $MgCl_2+6H_2O$ in 50 mL water. Sterile filter and store at 4 C. Label as "Stock 8: $MgCl_2$, date made".

Stock 9: Na pyruvate. Dissolve 0.220 g sodium pyruvate in 100 mL water. Sterile filter into an aluminum-foil wrapped 125 mL bottle and store at 4 C for 2 mo. Label as "Stock 9: Na Pyruvate, date made, expiration date".

Stock 10: Bovine Steer Serum (BSS). Prepare 10 mL aliquots of BSS (Pel-Freeze) and store at -20 C indefinitely. Label as "Stock 10: BSS, date made".

Stock 11: BSS/Hep. Add 1000 USP units of sterile heparin (dissolved in small volume of water) into 500 mL BSS. Store in 8 mL aliquots indefinitely at -20 C. Label as "Stock 11: FCS/Hep; date made" (5.34 mg).

Stock 12: Estradiol. Dissolve 1 to 3 mg estradiol in ethanol for a final concentration of 1 mg/mL. Store in a glass tube at -20 C for 2 months. Label as "stock 12: Estradiol, date made; expiration date".

Stock 13: FSH-P. Reconstitute FSH-P with saline for a final concentration of 5 mg/mL (follow direction on bottle). Place 500 μl aliquots into sterile 1.5 mL microfuge tubes and store indefinitely at -20 C. Label as "stock 13: FSH-P; date made".

Stock 14: Heparin. Dissolve 20 mg in 10 mL water. Pipette into 250 µl aliquots and store at -20 C in bullet tubes indefinitely. Label as "Stock 14: Hep, date made".

Stock 15: Gentamycin. (Sigma, G-1397; 50 mg/mL). Dilute to 5 mg/mL concentration with water and sterile filter. Pipette 1 mL aliquots into sterile 12 x 75 tubes and store at -20 C indefinitely. Label as "Stock 15: Gent; date made".

Stock 16: ABAM (1 mL). (Sigma, A-9909). Aliquot 1 mL into sterile 12 x 75 tubes and store indefinitely at -20 C. Label as "Stock 16: ABAM (1 mL); date made".

Stock 17: Pen/Strep (4 mL). (Sigma; p0781; 10,000 units Pen-G and 10 mg Streptomycin) Thaw 100 mL bottle and aliquot 4 mL into sterile 12 x 75 tubes and store at -20 C indefinitely. Label as "Stock 17: Pen/Strep (4 mL); date made".

Stock 18: ABAM (Home-made; 100X); for 10 l transport saline. Combine 0.555 g Amphotericin B (Solubilized; Sigma, A-9528; 25 ug/mL), 100 million IU Penicillin G (Sigma, P-3032; 10,000 IU/mL), and 100 g Streptomycin Sulfate (Sigma, S-9137; 10 mg/mL) with 10 L saline and allow for complete solubilization by prolonged stirring. Sterile filter or autoclave and store in 50 mL conical tubes in walk in freezer. Label as "Stock 18: Home-made ABAM (100X)". This stock can be used instead of Pen/Strep when there is a problem with fungus contamination.

Stock 19: Hyaluronidase. Prepare stock solution of type IV hyaluronidase at 10 mg/mL saline (approx. 8000 U/mL) and store 1.2 mL aliquots at -20 C indefinitely. Label as "Stock 19: Hyal.; date made".

Stock 20: PHE Mix. Prepare primary stocks of 1 mM hypotaurine (Sigma H-1348; 1.09 mg/10 mL saline), 2 mM penicillamine (Sigma P-5000; 3 mg/10 mL saline) and 250 µM epinephrine (Sigma E-4250; 1.83 mg/ 40 mL of the following solution (165 mg 60% Na lactate syrup, 50 mg Na metabisulfate and 50 mL H_2O)). Epinephrine is easily oxidized by direct light so take precautions to avoid this problem. Combine 5 mL of 1 mM hypotaurine, 5 mL of 2 mM penicillamine, 2 mL of 250 µM epinephrine and 8 mL of saline and sterile filter. Aliquot 400 µl of PHE Mix into sterile microfuge tubes and store in a light resistant container at -20 C indefinitely. Upon retrieval of PHE Mix for use, wrap tube in aluminum foil.

Stock 21: Glutamine. Prepare stock solution of 1.5 g glutamine/ 100 mL water, sterile filter and store 1 mL aliquots at -20 C indefinitely. Label as stock 21: Glut, date made.

Stock 22: $MgCl_2$ for Percoll. Prepare 0.1 M stock by adding 0.203 g $MgCl_2$ to 10 mL water. Sterile filter and store at 4 C indefinitely.

Stock 23: $CaCl_2$ for Percoll. Prepare 1 M stock by adding 0.735 g $CaCl_2+2H_2O$ to 5 mL water. Sterile filter and store at 4 C indefinitely.

Stock 24: Trypsin. Prepare 5 mL aliquots of 1x trypsin (Sigma, #T-5650) and store at -20 C until use.

Stock 25: 100 X Pen/Strep (Sigma; p0781; 10,000 units Pen-G and 10 mg Streptomycin) Thaw 100 mL bottles and prepare 10 mL aliquots. Store at -20 C until needed. Label as stock 25 100 X Pen/Strep (10 mL); date made.

Stock 26: 100 X ABAM (Sigma; A-9909) Thaw 100 mL bottles and prepare 10 mL aliquots. Store at -20 C until needed. Label as stock 26 100 X ABAM (10 mL); date made.

C. TCM-199 stock medium

This stock is used to prepare several types of media

Water	800 mL
TCM-199 (Earle's salts; powder) (Sigma, M-5017)	for 1 L
$NaHCO_3$	2.2 g

1. Adjust pH to 7.2 and bring volume to 1 l. Sterile-filter 90 mL aliquots into opaque or aluminum-foil wrapped 100 mL bottles and store at 4 C for 2 mo. Label as "TCM-199, -Suppl."; write date made on label.

2. Beginning 2 weeks after TCM-199 stock medium is made, 1 aliquot of stock 21 (glut.) is need to replenish glutamine that has degraded.

3. Alternatively, liquid medium can be purchased from Specialty Media, Inc. (Lavallette, NJ) catalogue number SLM-032-A or B.

D. Oocyte Collecting Medium

Water (use ddH_2O)	9 L
TCM-199 (-Phenol red, -Glut.) (Sigma, M-3274)	For 10 L
$NaHCO_3$	3.50 g
Stock 6: 1 M HEPES	100 mL

1. Adjust pH to 7.2 and bring volume to 10 L. Sterile-filter 400 mL medium into 500 mL bottles and keep indefinitely (?) at 4 C. Labels should read "Oocyte Collecting Medium, -BSS -Pen/Strep - Hep, date made".

2. On night before use, add 1 aliquot of stock 11: BSS+Hep, 4 mL glutamine and 1 aliquot of stock 17: Pen/Strep (4 mL) to one bottle. Change label to +BSS −Heparin +ABAM and use within 2 weeks. Write expiration date on label. Generally, 3 to 4 bottles are needed on the day of oocyte collection.

E. Oocyte Maturation Medium

1. On night before use, add 1 aliquot stock 10: BSS, 1 aliquot stock 15: gent., 400 µl stock 13: FSH-P, 200 µl stock 12: estradiol, 1 mL stock 9: Na Pyruvate and 1 mL stock 21: Glutamine to one bottle of TCM-199 (90 mL). Change label to read "Oocyte maturation medium" and use within 1 week. Write expiration date on label.

F. CZB medium: Early embryo culture medium (1-cell to 8-cell)

1. Prepare stock containing the following:

Ingredient	g/0.5 liter	g/liter
NaCl	2.385	4.770
Kcl	0.180	0.360
KH_2PO_4	0.080	0.160
$NaHCO_3$	1.055	2.110
EDTA	0.016	0.032
$MgSO_4+7H_2O$	0.145	0.290
$CaCl_2+H_2O$	0.125	0.250
Phenol Red	0.005	0.010

2. Adjust pH for 7.3 and sterile filter 88 mL of CZB medium into 100 mL bottles and store at 4 C indefinitely. Label as CZB medium, -Suppl.". Write date prepared on label.

3. On day before use add 0.45 mL stock 5: Na lactate, 1 mL stock 9: Na Pyruvate, 1 mL stock 21: Glutamine, 1 aliquot stock 15: Gent and 1 aliquot stock 10: BSS. Change label to read CZB medium + Suppl. and use within 2 weeks. Write expiration date on label.

G. Embryo Culture Medium

LEC

1. Add 1 aliquot stock 10: BSS, 1 aliquot stock 15: gent: 1 mL glutamine stock 21 and 1 mL stock 9 Pyruvate to 90 mL TCM-199. Change label to read "Late Embryo Culture Medium" and use within 2 weeks. Write expiration date on label.

CR1aa

Make CR1 stock (prepare in a 25 mL volumetric flask):

NaCl	167.50 mg
Kcl	5.75 mg
$NaHCO_3$	55.00 mg
Na Pyruvate	1.00 mg
Glutamine	3.75 mg
Hemi-Ca Lactate	13.75 mg

Add ~ 15 mL of culture grade water to volumetric flask and add first 5 ingredients to water. Thoroughly dissolve constiutents and then add Hemi-Ca Lactate. Note: constituents of this medium are known to precipitate out of solution. To minimize the chances of this occurring, use immediately after making. If a medium appears white and cloudy, discard and start again.

CR1aa (final working medium)

To 5 mL of CR1 stock add:

BSA Fraction V	3.00 mg/mL (15 mg)
Gentamycin Stock	0.5 µl/mL (2.5 µl)
Nonessential amino acids	50 µl (100 X stock)
Essential amino acids	50 µl (100 X stock)

Sterile filter medium; Rosenkrans and First 1991 35:p266 Theriogenology Abstr.

H. TL Media

Note: TL media are now made and purchased from Specialty Media.

Ingredient (mL) Catalogue Number	Sp-TL BSS-009-D	HEPES-TL BSS-011-C	IVF-TL BSS-010-D
Water	79.232	177.0	40.157
Stock 1: NaCl	4.34	10.0	2.5
Stock 2: KCl	1.96	4.0	1.00
Stock 3: $NaHCO_3$	10.00	1.6	5.00
Stock 4: PO_4	1.0	2.0	0.50
Stock 5: Na-lactate	0.368	0.372	0.093
Stock 6: HEPES	1.0	2.0	0
Stock 7: $CaCl_2$	1.0	2.0	0.50
Stock 8: $MgCl_2$	1.10	1.0	0.25
pH	7.4	7.3	7.4
Osmolarity (mOsm)	295-305	275-285	290-300

1. If media is prepared in laboratory, adjust pH on solutions and check osmolarity. If osmolarity is not correct, discard solutions and start over. Use TL stocks to prepare TALP solutions. Sterile filter remaining Sp-TL and store at 4 C for up to 1 week. Write expiration date of label.

I. TALP Media

Ingredient	Sp-TALP	Hepes-TALP*	IVF-TALP*
TL (mL)	38.0	100.0	50.0
BSA, Fraction V (mg)	240	300	0
BSA, EFAF (mg)	0	0	300
Stock 9: Na pyr (mL)	2.0	1.0	0.5
Stock 15: Gent (µl)	80	150	50
Stock 14: Hep (µl)	0	0	250

* Use entire volume of TL stocks to prepare TALP solutions

1. Sterile filter and store at 4 C until use. Discard after 1 week.

J. 90% Percoll

1. Purchase Percoll from Sigma (#P-1644) and store at 4 C until use.

2. Prepare 10x Sp-TL stock solution:

Chemical	g/100 mL H_2O
NaCl	4.675
Kcl	0.23
$NaH_2PO_4+H_2O$	0.40
HEPES	2.38

3. Adjust pH to 7.3, sterile filter and store indefinitely at 4 C.

4. To prepare 90% Percoll solution (on day of use), place 2 mL of 10x Sp-TL to a small beaker and add 0.042 g bicarbonate and 74 µl Na lactate (Stock 5). After bicarbonate and lactate dissolve, add 18 mL Percoll and stir.

5. While stirring, add 79 µl $MgCl_2$ (Stock 22) and 39 µl $CaCl_2$ (Stock 23).

6. Adjust pH to 7.3-7.45 and filter with a .45 um syringe filter. If compounds precipitate out of the Percoll solution, continue to stir. If compounds do not re-dissolve, then start over.

7. Prepare the 45% Percoll solution by adding equal volumes of 90% percoll solution and Sp-TL (1x). Mix thoroughly.

K. Hams-F12 medium

1. Prepare Hams-F12 medium (Sigma, #N-6760) by adding powder to ddH_2O (Simmen's Lab). Either 1 or 5 L of can be prepared, depending on rate of use. Add 1.176 g/l bicarbonate to solution, pH to 7.2 - 7.3 add bring up to the desired volume. Sterile filter into 100 mL or 500 mL bottles (again, depending on rate of use) and store at 4 C for up to 2 months.

2. On day of use, add BSS for a final concentration of 5% and 1% pen/strep. Use within 2 weeks.

3. If Hams-F12 stock is greater than 2 weeks old, add 1 mL glutamine per 100 mL medium.

L. Transport Saline

1. Prepare a carboy by determining 10 l mark.

2. Add 90 g NaCl and distilled water to the 10 l mark. Mix thoroughly. Label as "0.9% saline, date made" and store indefinitely at room temperature.

3. When needed pour saline into 1 L bottles and autoclave. When cooled add 10 mL stock 25: Pen/Strep to each liter of saline required. Pour ~700 mL into each of 2 sterile ovary buckets. Save remaining saline for washing ovaries following their transport to the laboratory.

Note: Adding ABAM when saline is too hot may destroy antibacterial and antifungal activities.

M. Mineral Oil

1. Purchase embryo tested mineral oil from Sigma (M-8410) and store at room temperature indefinitely.

2. Alternatively, paraffin (VWR, #S894-07) or silicon (Aldrich, #14,615-3) oil that has not been embryo tested can be used if first extracted with water. Combine equal volumes of oil and water and incubate at 37 to 40 C overnight. Repeat process two to three times and store oil at room temperature. However, toxic effects of these oils may still persist following extraction. Therefore, use of embryo tested mineral oil from Sigma is preferred.

N. Medium for Oviduct culture

Oviduct Culture Medium: Combine 1 aliquot stock 10: BSS, 1 aliquot stock 15: Gentamycin and 1 mL stock 9: Pyruvate. Store at 4 C and use within 1 week. If TCM-199 is more than 2 weeks old, add 1 mL glutamine (Stock 21) to bottle.

O. DAPI stain

1. Prepare PBS stock containing 3% glutaraldehyde (v/v; 3 mL glut./ 100 mL PBS). Store indefinitely at room temperature in opaque or foil wrapped bottle.

2. Prepare DAPI stock or use DAPI stock solution for determining embryo viability. Add 0.1 mg DAPI (Sigma, #D-9542) to 10 mL sterile saline (0.001% DAPI). Store at 4 C in foil wrapped culture tube for up to 4 months.

3. Add 200 µl stock 18: hyaluronidase to 800 µl maturation medium in microfuge tubes and place at room temperature.

4. Remove 10 to 20 oocytes from washing steps after maturation (day 2) or fertilization (day 3) and place in hyaluronidase solution for 2 to 3 min and centrifuge (via microfuge) for 1 minute.

5. Fix oocytes with 3% glutaraldehyde in a 35 x 10 mm culture dish at room temperature for 15 minutes.

6. Wash oocytes in PBS and transfer to 0.001% DAPI solution (in smallest volume possible; 500 µl). Incubate at 37 C for 10 minutes.

7. After DAPI staining, place oocytes on slides with a small volume of PBS (5 to 10 µl). DAPI will bind DNA and emit fluorescence with a 490 nm emission filter.

8. Oocytes have matured through meiosis (as desired) if staining is seen in a small area within the oocyte and within 1 polar body.

9. Oocytes have been fertilized when 2 pronuclei are evident.

APPENDIX B
ESTABLISHMENT OF PREGNANCY THROUGH TRANSFER OF IVM/IVF/IVC-DERIVED EMBRYOS TO ESTROUS SYNCHRONIZED COWS

Introduction

Pregnancy and delivery of live offspring following transfer of IVM/IVF/IVC embryos to recipient animals is commonly used as an indicator of embryo quality for a given culture system. The objective of this study was to determine if embryos produced using IVM/IVF/IVC procedures described in this thesis were competent to result in live calves after transfer to recipient females.

Procedures

Estrus was synchronized in 12 heifers using 2 injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 25 mg) given 14 d apart. Heifers were checked twice daily for estrus. Criterion used to assess estrual behavior was standing to be mounted; day of estrus was designated as d 0.

One day prior to predicted estrus, ovaries were obtained from the local abattoir. Oocytes were collected and allowed to mature for 24 h as previously described in Appendix A. Coincident with the day heifers displayed estrus, oocytes were cultured with sperm mixed from 3 bulls for 18-20 h. Thereafter, putative zygotes were denuded of cumulus and associated spermatozoa and placed in microdrops of LEC medium covered with mineral oil and containing 10-15 oviductal worms (10 putative zygotes/50 µl LEC; Appendix A). On d 3 post-IVF, cleavage rates were assessed and 50 µl of fresh LEC was added to each drop. Blastocysts were removed from culture drops on d 7 post-IVF and

washed once in Dulbecco's phosphate buffered saline (DPBS) containing 10% htFCS, 0.2 mM pyruvate and 50 μg/mL gentamicin prewarmed to 39°C, and loaded individually into prerinsed 0.25 mL French straws. Only expanded blastocysts were used for transfer to recipient females.

Embryos were transported to the farm in a 39°C thermos containing water. Animals were restrained and given an epidural injection consisting of 5 mL of Lidocaine. Thereafter, expanded blastocysts were transferred to the uterine horn ipsilateral to the corpus luteum (one blastocyst/heifer; n=5) following procedures detailed by Drost (1992). Pregnancy rates were determined using ultrasonography and palpation per rectum on d 32 and 69, respectively. Recipients that were diagnosed as pregnant were maintained until after delivery.

Results

Cleavage rate of IVM/IVF/IVC embryos was 64%. By d 32, 2 heifers were confirmed nonpregnant due to return of estrus. Ultrasonography on d 32 revealed the presence of an embryo in one heifer and a corpus luteum and fluid in the uterine space of the other two. Palpation per rectum on d 69 confirmed pregnancy in all 3 heifers. Each of these heifers gave birth to bull calves following gestation lengths of 277, 280 and 295 days, respectively. All calves were healthy at birth and clearly of different breeds. Calf number 2 later died of complications of a systemic infection. A photograph of the 2 surviving calves is shown in Figure B-1.

Figure B-1. Photograph of calves born as a result of transfer of IVM/IVF/IVC derived embryos to recipient females. The team involved in producing these calves were (from left to right) Dr. Peter Hansen, Susan Gottshall, Victor Monterroso, Carlos Aréchiga and Lannett Edwards.

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BIOGRAPHICAL SKETCH

Janice Lannett Edwards was born on November 11, 1966, in Camden, Tennessee, as the oldest daughter of William Lannie Edwards and Janice Hueline Alberd. At an early age, she developed an appreciation for agriculture. Her grandparents, William Durand and Ozella Edwards, owned and operated a dairy farm in Erin, Tennessee. In 1985, Lannett graduated from Houston County High School and was awarded a university service scholarship by Austin Peay State University. She obtained a Bachelor of Science degree in agriculture in 1989. While attending Austin Peay, Ms. Edwards was a little sister of Alpha Gamma Rho and a member of the Animal Science Club and the agricultural honor society, Delta Tau Alpha. In addition, she worked part time in a cafe as a waitress, cook and general manager, and as a veterinary assistant at the Animal Medical Hospital. With the guidance of Dr. Gaines Hunt, she developed an interest to further pursue studies at the graduate level in reproductive physiology of domesticated species.

In 1990, Ms. Edwards was awarded a graduate research/teaching assistantship in the Department of Dairy Sciences at Mississippi State University under the supervision of Dr. John Fuquay. Her thesis research investigated corpus luteum function and growth during summer and winter environments. While at Mississippi State, she developed a love and enthusiasm for science and instructing undergraduates. She actively taught various laboratories, gave lectures for several undergraduate and graduate level courses, and was chosen by the Dean to serve on a selection committee for the department head of

a newly merged Animal and Dairy Science Department. In her spare time, she worked with Dr. Nancy Cox to determine metabolic influences on preovulatory follicular growth in the pig. In 1991, she was elected to membership in the honor societies of Sigma Xi and Gamma Sigma Delta.

Following completion of her master's degree, Lannett was awarded a USDA National Needs Fellowship in 1993 and enrolled in the Animal Molecular and Cell Biology Interdisciplinary Graduate Concentration Program at the University of Florida. Under the supervision of Dr. Peter J. Hansen, she studied the ontogeny of thermotolerance in bovine embryos by specifically determining HSP70 synthesis and resistance to heat shock during oocyte maturation and preimplantation development and is now a candidate for the degree of Doctor of Philosophy. On April 9, 1996, Ms. Edwards was awarded the Graduate Student Research Award from the University of Florida Chapter of Sigma Xi. Upon completion of the Doctor of Philosophy degree, she will begin a postdoctoral fellowship with Dr. Caird E. Rexroad, Jr. at the U.S. Department of Agriculture in Beltsville, Maryland, in the Gene Evaluation and Mapping Laboratory. There she plans to study the culture of blastomeres with the intent to produce bovine embryonic stem cells.

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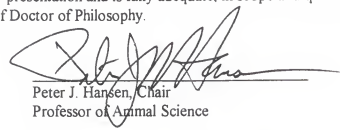

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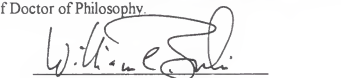

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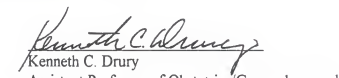

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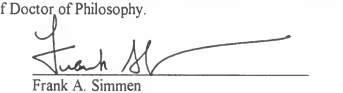

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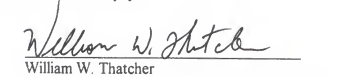

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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